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Warfarin: A Forgotten Rodenticide Primary and Secondary Effects of a Warfarin Bait for Black-Tailed Prairie Dogs

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WARFARIN: A FORGOTTEN RODENTICIDE

PRIMARY AND SECONDARY EFFECTS OF A

WARFARIN BAIT FOR BLACK-TAILED PRAIRIE DOGS

by

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Our goal was to assess warfarin as a candidate toxicant for the management of black-tailed prairie dogs (*Cynomys ludovicianus*) through primary and secondary toxicity laboratory tests.

The primary toxicity test entailed the use of 60 healthy adult prairie dogs, live trapped from a colony in eastern Colorado. Prairie dogs were assigned to 6 treatment groups; 0, 50, 100, 250, 500, and 1000 ppm warfarin bait with 5 males and 5 females in each treatment group. After a 10-day acclimation period, the prairie dogs were exposed each day to 70 grams of the respective bait for 15 days. Afterward, we maintained a 10-day post-test observation period, with 2 observations/day. Efficacy in the 500 ppm group was 100%. Interestingly, the 1000 ppm group had 80% efficacy. Total bait consumption was significantly different ($P < 0.000$) between the control and treatment groups, but the consumption among the treatments was not significantly different ($P = 0.05$). Similarly, weight loss from the ingestion of warfarin occurred in all treatment groups, but not in the control ($P < 0.05$).

To enhance the toxicity data of warfarin, a secondary hazard study was conducted to generate warfarin toxicity data by feeding warfarin-fed black-tailed prairie dogs to surrogate secondary animals, domestic ferrets (*Mustela putorius furo*). A combination of residue data in primary animals and dietary lethality in secondary animals was used to project potential field hazards. Black-tailed prairie dogs were fed warfarin bait for the secondary toxicity test because they represent a typical model of a target vertebrate pest in which warfarin baits will be used to manage populations.

Warfarin bait (500 ppm) was fed no-choice to black-tailed prairie dogs for 5 days. A 2-day post-test observation period followed and then prairie dogs were euthanitized with CO_2. Ten treatment and 2 control domestic ferrets (equal sexes in each group) were pre-conditioned to eating untreated prairie dogs for 3 days. Following the acclimation period, the ferrets were presented warfarin-treated prairie dog carcasses for 5 days. Observations were made on the ferrets' physical and behavioral health, and of the prairie dog anatomy consumed by the ferrets. Twenty-one days of post-test observation concluded the test. The domestic ferrets showed no signs of warfarin intoxication. No signs of weight change were evident between treatments of males ($\underline{P} = 0.380$) and females ($\underline{P} = 0.143$). The treated ferrets gained weight similarly to the control ferrets, and both treatment and control ferrets had similar consumption rates and feeding behaviors.

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Preface

The literature review, Part I, is to identify the current concerns and information associated with the black-tailed prairie dog to the reader. Many topics are covered in an effort to prepare the reader for the following 2 studies. Part II, "Laboratory Efficacy Study With 6 Warfarin Baits to Control the Black-tailed Prairie Dog" was written in the format of the "Proceedings of the 17th Vertebrate Pest Conference." This section of the thesis will be submitted for publication to this journal, and therefore, was written in the respective format. Part III, "Secondary Hazard of Warfarin-fed Black-tailed Prairie Dogs (*Cynomys ludovicianus*) to Domestic Ferrets (*Mustela putorius furo*)" was written to comply with the format of the journal "Mammalia." This section of the thesis will be submitted for publication to this journal, and therefore, was written in the respective format.

The appendices accompanying Parts I, II, and III of the thesis are used to further explain details of these sections. The 3 appendices are entitled, "Analytical Methods," "The Preparation of Black-tailed Prairie Dogs for the Secondary Hazard Toxicity Study," and "The Potential of Secondary Poisoning of When Exposed to Black-tailed Prairie Dogs Feeding on a Warfarin Bait." The black-footed ferret appendix was to estimate the possibility of toxic effects to a mustelid species, which is considered to be susceptible to the effects of anticoagulants. Information of similar species of mustelids was used to extrapolate the hazard of warfarin posed to black-footed ferrets, secondarily.

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PART I.

LITERATURE REVIEW OF WARFARIN AS A TOXICANT FOR THE BLACK-TAILED PRAIRIE DOG AND THE SECONDARY HAZARD TO DOMESTIC FERRETS

INTRODUCTION

Black-tailed prairie dog (*Cynomys ludovicianus*) numbers in the Great Plains were estimated at 5 billion in the early 1900's (Merriam 1902). At that time they were considered a pest to the ranchers and farmers because of their feeding on crops. Feeding by prairie dogs decreases plant biomass which could be used for consumption by domestic cattle (Taylor and Loftfield 1924, Hansen and Gold 1977, O'Meilia et al. 1982, Knowles 1986). Prairie dogs also serve as a reservoir of diseases that affect human and other wildlife (Barnes 1982). For these reasons, the prairie dog is still being eradicated with the use of toxicants and firearms, resulting in lower densities and widely scattered colonies (Clark et al. 1982, Foster and Hygnstrom 1990).

Prairie dog populations have declined considerably over the years due to widespread agriculture tillage and control programs. Farmers and ranchers continue to use control techniques to limit distribution of prairie dog and reduce damage on their land. Currently-registered toxicants have exhibited limited effectiveness and pose considerably hazards to the environment (Hygnstrom and Virchow 1994). I propose that warfarin be considered for federal registration as a prairie dog toxicant because it is a

cost-effective rodenticide, it is relatively nontoxic to birds (Christopher et al. 1984, Hagan and Radomski 1953), it degrades quickly in the gastro-intestinal tract (half-life of 42 hours) (Ford 1993), it is rapidly excreted from the body (Wong and Solomonraj 1980), animals often do not develop "bait shyness" (Meister 1995), and it is relatively safe with respect to secondary poisonings (Aulerich et al. 1987).

CONCERNS WITH PRAIRIE DOGS

Agriculture

Prairie dogs feed on several of the same species of grasses and forbs as livestock (Kelso 1939, Fagerstone et al. 1981, Uresk 1984). Prairie dogs decrease forage resources on ranches and farms by 18% on the pasture lands used by cattle (Hansen and Gold 1977). O'Meilia et al. (1982) stated that the herbage available to cattle was 33% and 37% less in plots occupied by prairie dogs compared to healthy, unoccupied mid- and tallgrass pastures in 2 concurrent years. In addition, losses from prairie dogs have been reported at 29% (Knowles 1986) and 80% (Taylor and Loftfield 1924).

Disease

Prairie dogs serve as a reservoir for *Yersinia pestis*, the bacterium responsible for bubonic plague (Barnes 1982, Barnes 1990). After an animal is infected, the disease can be transmitted directly or by arthropod vectors (Rice 1994, Barnes 1993). In areas where prairie dog colonies are adjacent to residential areas, the likelihood of contracting plague is greatly increased. Since 1975, 78% of the human plague cases have been acquired within a one-mile radius of the individual's home (Barnes 1982). Residents of the front

range area of Colorado are greatly concerned about plague because of the encroachment of residential areas into established prairie dog colonies, and the confirmation of plague near Longmont, Fort Collins, Boulder, and Denver (www.rsr.organization/plague, www.deltatech.com/eagleview, www.//128.138.12.27/ government /boulder_county/ other-current/plague.rel.). Human cases of plague have been reported in Colorado since 1941, with 2 cases in 1995, 3 in 1994 and 2 in 1993 (Custer 1995). Control may be needed in the future to manage this problem, and environmentally safe rodent control may have to be performed for the sake of pets and humans in the adjacent areas.

Environmental

The prairie dog ecosystem provides food and shelter for a variety of animals (Clark et al. 1982). Species richness increases with increased colony size and density (Reading 1993). The unique habitat that is promoted by prairie dogs, promotes grasses (Coppock et al. 1983 a, b) and herbivores (Hoogland 1995), which in turn attracts several species of carnivores (King 1959). O'Meilia et al. (1982) reported that small rodent numbers were tripled in plots associated with prairie dog colonies, compared to healthy mid- and tallgrass plots. Prairie dog colonies are important for burrowing owls (*Athene cunicularia*), because they supply as much as half of the owls' food during mid- and late-summer (Butts and Lewis 1982). Black-footed ferrets (*Mustela nigripes*) feed almost exclusively on prairie dogs, and require the habitat provided by prairie dog colonies (Powell et al. 1985, Cully 1993).

The importance of the prairie dog as a keystone species is evident (Foster and Hygnstrom 1990), but petitions for endangered species listing have failed (US Fish and

Wildlife Service 1994). Up to 163 species of animals have been associated with prairie dog ecosystems (Clark et al. 1982, Agnew et al. 1986, Reading et al. 1989, Sharps and Uresk 1990). The mountain plover (*Charadrius montanus*, Knowles et al. 1982), ferruginous hawk (*Buteo regalis*, Cully 1991) and swift fox (*Vulpes vulpes*, Uresk and Sharps 1986) are possible additions to the jurisdiction of the Endangered Species Act.

Social

Some of the general public, including animal welfare and animal rights activists, have pushed for the halt of prairie dog control. Increasing sentiment is being gained for the prairie dog and some people believe non-lethal means of management are necessary (Grantier 1996). Opponents to the activists, however, still remove or control the prairie dogs. Recently, Gay Balfour, of Cortez, Colorado, modified a sewer pump truck to suction up prairie dogs from their burrows, causing little physical harm to the prairie dogs, and then moved them to another location (Grantier 1996). Translocation of prairie dogs, however, has been shown to be relatively unsuccessful (Robinette et al. 1995).

PRAIRIE DOG CONTROL

Toxicants

In the late 1800s, the use of strychnine, bisulfide of carbon, cyanide of potassium, phosphorus, sodium monoflouroacetate, zinc phosphide and the plow reduced the prairie dog distribution to 40% of their original range (Merriam 1902, Nelson 1919, Anderson et al. 1986). Government poisoning programs in the early 1900s resulted in further

reductions (Bell 1921). Drastic fragmentation of the distribution of prairie dogs resulted, leaving only 2%-5% of the original distribution (Anderson et al. 1986).

Strychnine and arsenic, were used for prairie dog control in the 1900s (Merriam 1902). However, because of their potential for secondary poisoning, toxicity to humans, and problems with bait acceptance, they fell out of public favor and lost their EPA registration. In addition, strychnine and arsenic were used for commensal rodents, but were eventually replaced by warfarin, one of the first anticoagulants for rodent control (Ford 1993).

Current Control

The only toxic baits that are currently registered for control of prairie dogs incorporate zinc phosphide as the active ingredient (Hygnstrom and Virchow 1994). These baits are effective in the control of prairie dogs (Schoof 1970, Dimmick 1972, Hood 1972, Uresk and Sharps 1986, Apa et al. 1990), but there are some negative effects. First, in field situations, zinc phosphide bait may remain toxic for many months because mineral oil carriers protect it from weather degradation (Timm 1994). Second, it can cause primary poisoning in birds and rabbits (Hegdal and Gatz 1977, Savarie 1991) Third, secondary consumption by predators or scavengers may lead to death of protected species such as the black-footed ferret if undigested zinc phosphide bait is consumed (*Mustela nigripes*). The siberian ferret (*Mustela eversmanni*) has shown some toxicity signs associated with zinc phosphide ingestion (Stroganov 1962, Hill and Carpenter 1982). In the Hill and Carpenter toxicity study (1982), emesis was noticed in three of the ferrets, which is a common characteristic of zinc phosphide acute intoxication. In the

same study, they showed that sublethal doses of zinc phosphide (2%) caused altered blood chemistries in the ferrets, which has been associated with damage of the liver, kidney, and heart tissue (Chitty 1954, Stephenson 1967, Janda and Bosseova 1970). Straube et al. (1980), reported that ferrets are more susceptible to zinc toxicosis than other species. Ferrets at a New Zealand ferret farm suffered dietary zinc toxicosis (postmortem liver analysis 203 to 881 ppm) (Buck et al. 1976). Another study reported zinc phosphide as having no secondary effects on domestic ferrets (Matschke et al. 1992), but no blood chemistry analyses were performed.

Two fumigants, aluminum phosphide and gas cartridges are also registered for the control of prairie dogs (Jacobs 1994). Aluminum phosphide is an acute toxin that produces effective control if properly used (Salmon et al. 1982, Hygnstrom 1994). Its effects, however, are non-specific. The product label directs that pellets of the toxicant must be placed into active prairie dog burrows, and then the burrow openings are covered with soil. The toxicant reacts with the humidity in the air and forms phosphine gas (PH_3), which is highly toxic, and it permeates throughout the burrow system. Any animal within the burrow system will most likely succumb to the phosphine gas (Meister 1996). The burrows may include many different non-target avain, mustelid, lagamorph, and rodent species (Hoogland 1995). Similarly, gas cartridges are used to fumigate burrows by lighting a fuse and throwing the cartridge into the burrow and then covering the burrow openings with soil. Combustion of the cartridge produces toxic gases including sodium nitrate, carbon monoxide, carbon dioxide, and other noxious gases. The gas travels throughout the burrow killing anything within the burrow's confines. Scientists are still searching for prairie dog toxicants that are cost-effective and environmentally safe. A

review of the current literature leads to the idea of the development of warfarin as an alternative toxicant for the management of prairie dogs. The use of warfarin as a rodenticide is widespread and its safety, efficacy, and availability are well known.

WARFARIN AS A CONTROL DEVICE

History

In the 1920s, a hemorrhagic syndrome in cattle developed in North Dakota and Canada (Schofield 1923, Roderick 1929). It was later associated with the consumption of improperly cured or moldy sweet clover hay (Buck et al. 1976). Ten years later, the hemorrhagic syndrome was found to be caused by a prothrombin deficiency, a primary protein precursor in blood clotting, caused from coumarin in the hay (Campbell et al. 1941). During the 1940s, K. P. Link and associates, with the Wisconsin Agricultural Experimental Station synthesized the causative agent bishydroxycoumarin. Warfarin ($C_{19}H_{16}O$) was first patented by the Wisconsin Alumni Research Foundation (W.A.R.F.) (Campbell and Link 1941).

Anticoagulant baits are the most frequently used vertebrate control device in the world (Clarke and Clarke 1967). Warfarin baits have effectively controlled animal populations since they were first developed in the 1940s (Buck et al. 1976). Warfarin has gained the advantage in the market place over the acute toxicants since its introduction in 1949 (Prier and Derse 1962). Warfarin baits have been used and proven effective on a variety of species including: nutria (*Myocaster coypus*) (Evans and Ward 1967), rat (*Rattus spp.*) (Hagan and Radomski 1953), and house mouse (*Mus musculus*) (Bentley and Larthe 1959). Warfarin has been therapeutically used for thrombotic disorders and

coronary disease in humans (Buck et al. 1976). Noteable individuals such as President Eisenhower have been treated for heart problems with warfarin (Harte et al. 1991).

Following many years of baiting with warfarin, some isolated populations of Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus*) appeared to develop resistance to warfarin (Rowe and Redfern 1968, Hermodson et al. 1969, Jackson and Kaukeinen 1972, Bishop and Hartley 1976, Leck and Park 1981). Individual survival may have been facilitated by genetic coding that reduced enzyme reactivity to warfarin, reversed the warfarin activity with an increased utilization of vitamin K, and enhanced the animals' ability to metabolize vitamin K (K_1) from menadione (K_3) in the liver (Thijssen 1995, MacNicoll 1995).

As a response to rodent resistance, other anticoagulant compounds were developed and marketed that were much more toxic, requiring one-fifth the active ingredient. These compounds were nicknamed the "second generation" anticoagulants which included other hydroxycoumarin compounds, (brodifacoum and bromadiolone) and others in the indandione group (chlorophacinone and diphacinone) (Ford 1993).

Some of these new compounds also have been tested on the prairie dog in the laboratory. Fisher and Timm (1988) tested chlorophacinone which proved to be effective in the primary use, but also was highly toxic secondarily to domestic ferrets (*Mustela putorius furo*). Bromadiolone was tested on prairie dogs in a lab situation and proved to be 100% effective for the control of prairie dogs. When the prairie dogs were fed secondarily to domestic ferrets, no deaths occurred (Fisher et al. 1991).

Human Safety

Warfarin has been proven to be an effective toxicant for rodent control, and it is relatively safe to humans. One human warfarin poisoning occurred after a male had taken one 114 gram container of "d-Con" for 6 continuous days (Holmes and Love 1952). He was treated with vitamin K and was released 13 days later after prothrombin times were again normal. In addition, a 14-member family in Korea subsisted on a warfarin corn meal bait for 15 days (Lisella et al. 1971). The family consumed 1-2mg/kg body weight/day, and all but two of the family members survived after vitamin K treaments. The warfarin treatment period is about 70% less than that for difenacoum and chlorophacinone, showing the relative safety of warfarin in human poisoning situations.

Difenacoum, brodifacoum, chlorophacinone, and bromadiolone, all second generation anticoagulants, have a 100 times stronger diminution of vitamin K-dependent clotting factor synthesis than warfarin (Lipton and Klass 1984). Cases of difenacoum and chlorophacinone human poisonings have resulted in vitamin K and plasma-product treatments for 42 days and 45 days, respectively (Jones et al. 1984, Murdoch 1983).

Secondary Hazard

Deaths caused from secondary exposure to warfarin have occurred, but in comparison to the acute toxicants and second generation anticoagulants, warfarin is a much safer compound.

Prier and Derse (1962) reported that warfarin was lethal to dogs ingesting 0.2 ppm/day, but 2.5 ppm/day secondarily through mice presented no effects. The primary and secondary effects of warfarin can be extrapolated from intoxication data from similar

species, but this should be done with caution, for different species can show dramatically different results (Weser and Sellers 1971). Although potential secondary hazard can be estimated, its actual existence with warfarin has not been proven in field situations (Holler and Lefebvre 1981). Appendix 3 will further address the possiblity of secondary toxicosis to black-footed ferrets.

Secondary poisoning of tawny owls (*Strix aluco*) was investigated by Townsend et al. (1981). Although 90% of the warfarin in the diet was absorbed by the owls, excretion and tissue residues accounted for only 10% of the consumed warfarin. Liver function within birds can metabolize warfarin into harmless conjugates very quickly. Within one hour, the consumed portion of warfarin was metabolized down to 60-70% of the initial amount ingested (Townsend and Tarrant 1977). Evidence suggests that birds are not sensitive to primary warfarin toxicity (Rudd and Genelly 1956, Papworth 1958, Bailey et al. 1973, Jones and Townsend 1978). Fumarin, a compound similar in its toxicity to warfarin with the same dosage, showed no apparent intoxication to barn owls (*Tyto alba*) when fed fumarin-killed rats (Mendenhall and Pank 1980).

Domestic ferrets were used as a model to detect secondary hazard to species of weasels and black-footed ferrets that may be found in prairie dog colonies (Fagerstone 1987). Least weasels (*Mustela nivalis*) are considered at high risk from the secondary consumption of warfarin-poisoned individuals because they have high metabolic rates, high food intake rates, and high susceptibility to cold weather stress (Townsend et al. 1984). The secondary consumption of warfarin-poisoned mice caused death in least weasels at a feeding rate of approximately 0.3 ppm/day for 10 days (Townsend et al. 1984). The secondary consumption of warfarin-poisoned rabbits that causes death in

mink (*Mustela vison*) is >3.0 ppm/day for 28 days (Aulerich et al. 1987). Within the same study, an LC_{50} value of 11.7 ppm was determined for mink. If least weasels could have a constant exposure and a minimum daily intake of 0.3 mg warfarin/kg body weight, death could occur. Mink never displayed any signs of poisoning from the effects of warfarin. Susceptibility to anticoagulants can be exacerbated by changes in diet (Colvin and Wang 1974, Laliberte et al. 1976) or increased activity (Oliver and Wheeler 1978, Penumarthy and Oehme 1978).

CONCLUSION

The need for prairie dog control has been demonstrated, but the value of prairie dog ecosystems precludes extermination. Currently registered prairie dog toxicants have several disadvantages relative to efficacy and human and environmental safety. Warfarin has been shown, by case study, to be very safe primarily to representative birds and safe secondarily to representative mustelids. Warfarin has been used beneficially in agriculture and urban situations. A warfarin bait could be an alternative toxicant for the control of prairie dogs. With judicious use of warfarin bait, substantial control could be expected with low non-target hazards.

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PART II.

LABORATORY EFFICACY STUDY OF SIX WARFARIN BAITS FOR CONTROLLING BLACK-TAILED PRAIRIE DOGS

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ABSTRACT: Control of the black-tailed prairie dog (Cynomys ludovicianus) is important for the reclamation of rangeland for cattle and limiting the spread of disease to humans and other wildlife. Six different concentrations of warfarin bait (0, 50, 100, 250, 500, and 1000 ppm) were fed to prairie dogs to determine efficacy. By day 10 of the exposure period, 49 of 50 prairie dogs were showing signs of warfarin intoxication. The 44.8 and 89.5 ppm groups had 30% and 50% mortality. The higher treatment groups, 233.0, 407.0, and 777.6 ppm, had mortality of 60%, 100%, and 80%, respectively. The lower treatment levels failed to cause sufficient trauma to cause death, while all but 2 prairie dogs were susceptible to the 2 higher treatment levels. The calculated LC_{50} for the warfarin baits is 97 mg/kg body weight. The concentrations consumed was correlated to the treatment group dosage ($r = 0.916$). The body weights of the prairie dogs decreased after warfarin exposure in all treatments except the control (HSD = 1.000, $P < 0.05$). The control group was the only group in which body weight increased. The whole body tissue analysis concentrations of the prairie dogs was correlated to the increase in treatment group dosage ($r = 0.709$). In this laboratory study, the 407.0 and 777.6 ppm warfarin baits were shown to be effective in the control of the black-tailed prairie dog. The lower treatment levels do produce mortality, but to an extent that survival rates are unacceptably high. For effective control, warfarin bait should be applied in early spring, when prairie dogs are stressed with low body weight from the over winter loss of fat reserves, and when forage is relatively low in biomass and nutritional quality. With suppressed amounts of dietary vitamin K, the prairie dogs are more susceptible to the warfarin bait.

KEY WORDS: Cynomys ludovicianus, black-tailed prairie dog, toxicant, rodenticide, warfarin, bioassay

INTRODUCTION

Black-tailed prairie dog (Cynomys ludovicianus) numbers in the Great Plains were estimated at 5 billion in the early 1900's (Merriam 1902). Historically, they were considered to be a pest to the ranchers and farmers because of their feeding on crops by the early settlers (Foster and Hygnstrom 1990). Grazing biomass has been decreased by the feeding action of prairie dogs (Taylor and Loftfield 1924, Hansen and Gold 1977,

O'Meilia et al. 1982, Knowles 1986). Prairie dogs also serve as a reservoir for diseases that can be transmitted to humans (Barnes 1990). For these reasons, prairie dogs populations are still being managed, primarily by the use of toxicants (Hygnstrom and Virchow 1994), resulting in lower densities and widely scattered colonies (Clark et al. 1982; Foster and Hygnstrom 1990).

In the late 1800s, the use of strychnine, bisulfide of carbon, cyanide of potassium, phosphorus, sodium monoflouroacetate, zinc phosphide and the plow reduced the prairie dog distribution to 40% of their original range (Merriam 1902, Nelson 1919, Anderson et al. 1986). Government poisoning programs in the early 1900s resulted in further reductions (Bell 1921).

Strychnine and arsenic, were used for prairie dog control in the 1900s (Merriam 1902). However, because of their potential for secondary poisoning, toxicity to humans, and problems with bait acceptance, they fell out of public favor and lost their EPA registration. In addition, strychnine and arsenic were used for commensal rodents, but were eventually replaced by warfarin, one of the first anticoagulants for rodent control (Ford 1993).

The only toxic baits that are currently registered for control of prairie dogs incorporate zinc phosphide as the active ingredient (Hygnstrom and Virchow 1994). These baits are effective in the control of prairie dogs (Schoof 1970, Dimmick 1972, Hood 1972, Uresk and Sharps 1986, Apa et al. 1990), but there are some negative effects. First, in field situations, zinc phosphide bait may remain toxic for many months because mineral oil carriers protect it from weather degradation (Timm 1994). Second, it can cause primary poisoning in birds and rabbits (Hegdal and Gatz 1977, Savarie 1991)

Third, secondary consumption by predators or scavengers may lead to death of protected species such as the black-footed ferret if undigested zinc phosphide bait is consumed (*Mustela nigripes*). The siberian ferret (*Mustela eversmanni*) has shown some toxicity signs associated with zinc phosphide ingestion (Stroganov 1962, Hill and Carpenter 1982). Straube et al. (1980), reported that ferrets are more susceptible to zinc toxicosis than other species. Ferrets at a New Zealand ferret farm suffered dietary zinc toxicosis (postmortem liver analysis 203 to 881 ppm) (Buck et al. 1976). Another study reported zinc phosphide as having no secondary effects on domestic ferrets (Matschke et al. 1992), but no blood chemistry analyses were performed.

Two fumigants, aluminum phosphide and gas cartridges are also registered for the control of prairie dogs (Jacobs 1994). Aluminum phosphide and gas cartridges produce effective control if properly used (Salmon et al. 1982, Hygnstrom 1994). Their effects, however, are non-specific. The product label directs that aluminum phosphide pellets or gas cartridges of the toxicant must be placed into active prairie dog burrows, and then the burrow openings are covered with soil. The toxicants form highly toxic gases, which permeate throughout the burrow system. Any animal within the burrow system will most likely succumb to the gases (Meister 1996). The burrows may include many different non-target avain, mustelid, lagamorph, and rodent species (Hoogland 1995). Scientists are still searching for prairie dog toxicants that are cost-effective and environmentally safe.

Fisher and Timm (1988) tested chlorophacinone on prairie dogs which proved to be effective in the primary use, but also was highly toxic secondarily to domestic ferrets (*Mustela putorius furo*). A review of the previous study and current literature lead to the

idea of the development of warfarin as an alternative toxicant for the management of prairie dogs because of its similar primary toxic effects, but a safer secondary toxicity. The use of warfarin as a rodenticide is widespread and its safety, efficacy, and availability are well known.

Even with the widely scattered colonies of prairie dogs, farmers and ranchers continue to use control techniques. I am proposing warfarin as a possible tool, because it is relatively nontoxic to birds (Christopher et al. 1984; Hagan and Radomski 1953), it quickly degrades in the gastro-intestinal tract (half-life of 42 hours) (Ford 1993), it is rapidly excreted from the body (Wong and Solomonraj 1980), animals often do not develop "bait shyness" (Meister 1996), and it is relatively safe with respect to secondary poisonings (Aulerich et al. 1987). Our goal was to identify the concentration of warfarin that would be the most effective level in a bait formulated for black-tailed prairie dogs. Specific objectives were to determine the mortality, illness, and weight loss associated with 6 concentrations of warfarin.

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METHODS

Warfarin Formulations

Six formulations of warfarin bait (0, 50, 100, 250, 500, and 1,000 ppm) were produced using warfarin technical product (99% purity) supplied by Sigma Chemical Company, St. Louis, Missouri. Initially, 3 warfarin concentrates (5,000, 10,000, and 20,000 ppm warfarin) were made to aid in obtaining a sufficient homogeneity for the warfarin formulations. After each ingredient was added to the grain, the mixer was operated for 20 seconds to mix the ingredients together. When large amounts of the concentrate were to be added (10,000 and 20,000 ppm groups), small portions of the concentrate would be added and then mixed for about 20 seconds. After all of the ingredients were added, the mixer was run for 15 minutes to achieve a homogenous mixture. The control bait, which contained 0 ppm warfarin, was mixed in the same manner as the other formulations. The exact ingredients cannot be stated for reasons of confidentiality.

All baits were analyzed to determine exact warfarin levels. Approximately 1, 40 gram sample was collected from each of the 6 formulations for freezer storage stability analyses, animal room stability analyses, and concentration verification analyses. Exact methods of the extraction procedure and analyses are presented in Appendix I. Bait samples were ground using a Udy Cyclone Sample Mill (Udy Corporation, Fort Collins, CO). Methanol was used to extract the warfarin from the bait matrix. The solvent and the bait were taken through 2 series of sonication, shaking, centrifuging, and decanting, before the warfarin extraction was complete. The analyte was then placed into a vial and analyzed by High Performance Liquid Chromatography (HPLC) for warfarin

concentration. Analysis of the freezer storage samples was performed after the exposure period to determine loss or degradation of the warfarin in the freezer. Samples were taken on the day of mixing or 2 days before the initiation of the 15-day exposure period. Analysis of the animal room stability samples was performed after the exposure period to determine loss or degradation of the warfarin in the environment of the animal room. Samples were taken on the day of mixing or 2 days before the initiation of the exposure period.

Test System

All methods used in the study were approved by the Genesis Laboratories, Inc. Institutional Animal Care and Use Committee (Project #96018). I used Tomahawk live traps (Tomahawk Live Trap Company, Tomahawk, Wisconsin) to capture 66 black-tailed prairie dogs from a colony in Larimer County, Colorado. Traps were set near the burrow openings and worked into the ground to cover the metal bottom with soil. Clean rolled barley was placed on the trigger device as well as a path leading out of the trap for a distance of about 0.5 meters. Traps were checked at least twice daily.

The prairie dogs were transported to Genesis Laboratories, Inc. (Wellington, Colorado) in the back of a covered truck in the same trap in which they were captured. All animals were dusted with Adams™ Flea and Tick Dust II (0.05% pyrethrin, SmithKline Beecham Animal Health, West Chester, Pennsylvania) to control ectoparasites.

I used "Ran30" (Faisal Ahmed, Colorado State University, Fort Collins, Colorado) a random number generating computer program to assign animals to treatment groups.

Prairie dogs were kept in 2, 500 gallon stock tanks for approximately an 60 minutes according to sex prior to assignment to treatment groups and individual cages. Sixty prairie dogs were randomly choosen from the stock tanks, weighed, and then randomly assigned to individual cages in 1 of the 6 treatments 1 day before the acclimation period.

Individual prairie dogs were placed into a cloth bag and plastic container and weighed. Prairie dog maturity was assessed according to body weight (minimum 675 grams and 775 grams for adult females and adult males, respectively) (Hoogland 1995). Preliminary body weights were recorded on the first day of the 10-day acclimation period when the prairie dogs were assigned to treatment groups. A final assessment of maturity by body weight was taken on Day 0, the first day of bait exposure. Underweight animals, based on the above criteria, were replaced with extra animals of sufficient weight. Final body weights were recorded at test termination or at time of death.

The individual cages had metal screen bottoms with a surface area of 3,720 cm^2 and a height of 41 cm (National Research Council 1992). Prairie dogs received a basal diet of Rodent Laboratory Chow® 5001 (Purina Mills Inc., St. Louis, Missouri), rolled barley, and water *ad libitum*. Bedding and water bottles were changed weekly, while water and feed levels were checked daily. Cages and racks were not cleaned during the study because handling of the animals could cause lesions, bruises, or injury that could bias mortality estimates (Penumarthy and Oehme 1978).

We recorded the minimum/maximum temperature and humidity of the animal room daily during the entire holding period with a calibrated digital hygrothermometer. Conditions in the study room was maintained at 16 to 26°C and 11 to 41% relative humidity. Air venting was checked weekly to maintain at 10 to 15 air exchanges per

hour.

The prairie dogs were acclimated to test conditions for 10 days before administration of the warfarin bait. The feeding-test period was conducted for 15 days, during which the 6 formulations were presented to each of the respective treatment groups until the end of the test period or death. Seventy grams of each formulation was presented daily in stainless steel feed cups. The feed cups were attached to a 30 cm x 30 cm sheet of particle board to retain spilled feed and stabilize the feed cup. A flat circular fowler with 10 mm holes was placed over the bait to limit spillage. Bait consumption was measured and recorded daily. After the exposure period, the prairie dogs were fed the basal laboratory diet *ad libitum* and observed for 10 days for signs of warfarin toxicity.

The prairie dogs were observed daily during the entire holding period. Physical or behavioral signs were recorded daily to determine the onset of illness or anticoagulant poisoning during the test. Symptoms included: diarrhea, ataxia, immobility, hemorrhage, hyperreactivity, hyporeactivity, bloody stool, labored breathing, hind limb paralysis, moribundity, and death. Prairie dogs were observed once each day during the 10-day acclimation period, and twice daily during the 15-day feeding-test and 10-day post-test observation periods. The United States Environmental Protection Agency (EPA) recommended the 15-day feeding test and a 5-day post-test observation period (William Jacobs pers. comm.) We used a 10-day post-test period to be certain no mortality went unobserved. Necropsies were conducted on all animals that died during the test. Animals were incised from the anus to the lower jaw. All major organs were observed for hemorrhaging and signs of anticoagulant poisoning in the abdominal and thoracic

regions.

Any prairie dogs found dead during the test were labeled, wrapped in foil, and stored in a freezer for later analysis. We randomly selected prairie dogs from the freezer, 3 from each of the 50, 250, and 1,000 ppm treatment groups, for whole body warfarin residue analysis. The frozen prairie dogs were thawed in 5 gallons of warm water and then blended with an equal amount of acetone until homogenized (1 g prairie dog : 1 ml of acetone). We collected 3, 5 g samples from each homogenized prairie dog, diluted with acetonitrile, and then minced the samples in a Tissumizer (Tekmar Corporation, Cincinnati, Ohio). The samples were centrifuged, and the analyte was decanted into clean centrifuge tubes. The extraction procedure was performed twice. The extracts were analyzed by HPLC (Appendix I).

SPSS for Windows, release 7.5 (November 14, 1996, Standard Version, Copyright © SPSS Inc. 1989-1996) was used to analyze the data. Relationships between dependent (mortality, weight loss, and consumption) and independent variables (treatment level) were compared using linear regression. Levene's test for homogeneity of variances was used to compare the variances among treatment groups. I used analysis of variance (ANOVA) and Kruskall-Wallis tests to compare treatment group means when the variances were or were not homogeneous, respectively. The Tukey's honestly significant difference (HSD) test was used to identify significant differences among the means of the treatment groups ($\alpha = 0.05$).

The computer program "Toxstat, version 3.4" (West, Inc. and Dave Gulley, University of Wyoming, Cheyenne, Wyoming) was used to calculate the LC_{50} and LC_{90} values for the warfarin formulations. This program uses the number of animals that died

in each of the treatment groups and calculates the concentration required to cause death in a percent of the population. It calculates the lethal concentration (LC) values in increments of 10%. LC_{50} and LC_{90} values represent the calculated concentration it takes to cause death in 50% and 90% of the sample population of prairie dogs, respectively.

RESULTS

The warfarin baits used in this study were formulated to be nominal concentrations of 0, 50, 100, 250, 500, and 1000 ppm. The validated analytical extraction procedure resulted in warfarin baits of actual concentrations of 0.0, 44.8, 89.5, 233.0, 407.0, and 777.6 ppm. For the remainder of the report, the actual concentrations will be used for all calculations and presented results.

During the first 5 days of the exposure period, only 1 female prairie dog showed signs of lethargy affiliated with warfarin intoxication (233.0 ppm treatment group). By day 10, 49 of 50 prairie dogs began showing signs of hemorrhaging (nose, mouth, anus) or lethargy, with increasing severity as the study progressed. The first death occurred on day 8 of the exposure period, in the 233.0 ppm group. By day 15, the severity of the symptoms continued to progress with 16 and 18 of 50 individuals becoming moribund or found dead, respectively.

After the 15-day feeding test period, the animals were observed for 10 more days in a post-test observation period. Fifteen of 32 animals continued to deteriorate physically, while the other 17 began to show improvements in their physical condition or showed no signs of anticoagulant poisoning. In an extreme case, animal number M23, in the 233.0 ppm treatment group, was classified as being "moribund" for 13 days, and then

was upgraded to lethargic on day 8 of the post-test observation period. He survived for the duration of the test. Prairie dogs in the 3 highest treatment groups were dying from warfarin toxicosis up to 7 days after the last death in the 2 lower treatment groups. Nine of 20 prairie dogs in the 2 lower groups recovered from the warfarin exposure completely, and 3 others had decreasing severity of symptoms, showing recovery on the last day of the test. The 44.8 and 89.5 ppm groups had 30% and 50% mortality. The higher treatment groups, 233.0, 407.0, and 777.6 ppm, had mortality of 60%, 100%, and 80%, respectively.

Mortality rates varied from 30% to 100% for the 5 warfarin treatment levels (Table 1). No difference among sexes was observed. Interestingly, the 777.6 ppm group did not produce 100% mortality like the 407.0 ppm treatment group below it. One male and 1 female survived, although they lost 24 and 38 grams more than the mean male and female weight loss, respectively. Further investigation revealed that the male ate only 128.6 mg of warfarin/kg body weight and lost 146 grams of body weight. The female prairie dog consumed 186.1 mg of warfarin/kg body weight and lost 267 grams of body weight. The male and female consumed 121.9 and 43.3 mg warfarin/kg body weight less than the mean of each sex, respectively.

The mean body weight of prairie dogs at the beginning of the exposure period was 897 grams. Mean body weights of prairie dogs across treatments were similar ($F = 0.657$, $\underline{P} = 0.658$) as were the variances ($L = 0.545$, $\underline{P} = 0.741$). The body weights of the animals decreased after warfarin exposure in all treatments except the control (Table 2). Treatment means differed ($\underline{P} < 0.000$) according to ANOVA. Weight loss from consumption of the treated baits was significantly different from consumption of the

untreated control bait (HSD = 1.000, $P < 0.05$) while no difference was evident between the treated groups (Figure 1). Data among the treatment groups were normally distributed and variances were homogenous ($L = 1.497$, $\underline{P} = 0.208$).

The necropsies showed small to large amounts of hemorrhaging, independent of the level of warfarin treatment. Control animals were not necropsied because I did not want to unnecessarily kill animals. During and after the exposure period, hemorrhaging was observed in the nose, eye, mouth, stomach, liver, intestines, cecum, kidneys, anus, heart, lungs, brain, and subcutaneous and neck regions. The hemorrhaging was so extensive in 7 animals that blood pooled in the abdomen (5), thorax (1), or subcutaneous tissue (1). Twenty-nine of 32 prairie dogs that died during the test were observed to have extensive fat reserves in the abdominal region. Fat was also present in the thoracic region in 25 of 32 prairie dogs. No sex-specific differences were observed according to treatment level.

Bait consumption differed among treatment groups ($\underline{P} < 0.000$), but not between sexes ($\underline{P} = 0.469$), as indicated by ANOVA. Consumption differences occurred between the control group and all treatment groups, and only small differences occurred among the treatment groups (HSD = 0.430, $\underline{P} < 0.05$) (Figure 2). The data were normally distributed and variances were homogeneous ($\underline{P} = 0.128$). Warfarin consumption was highly correlated ($r = 0.916$) with the concentration of warfarin baits presented (Figure 3). A Kruskall-Wallis test indicated that the treatment means were significantly different ($\underline{P} < 0.000$). Consumption of warfarin in the 2 highest treatment levels was greater than the 3 lower treatment levels. The data were normally distributed but variances were not homogenous ($\underline{P} < 0.000$).

The computer program "Toxstat, version 3.4" produced an LC_{50} value of 97 mg/kg body weight for black-tailed prairie dogs and an LC_{90} for black-tailed prairie dogs of 831 mg/kg body weight. Other analyses were calculated because of the 80% efficacy achieved in the 777.6 ppm treatment group. One analyses was performed without the 777.6 ppm treatment group and another with 100% efficacy in the 777.6 ppm group. All other treatment level variables remained the same. These alternative analyses showed similar calculated LC_{50} values of 94 and 93 mg/kg body weight, respectively. The LC_{90} values were much lower than the original value, 441 and 376 mg/kg body weight, respectively.

Mean warfarin concentrations extracted from the tissues of prairie dogs that expired during the test were 0.27, 1.18, and 3.11 ppm in the 44.8, 233.0, and 777.6 ppm treatment groups, respectively (Table 3). Warfarin accumulation was correlated with the warfarin levels in the treatments ($r = 0.709$). Also, using a validated laboratory method for the analysis of bait samples, concentration verification, freezer storage stability, and animal room stability samples of all warfarin concentrations were analyzed. All types of samples analyzed showed similar values respective to the treatment levels of the baits. Results of the test are presented in Appendix I, Tables 3, 4, and 5, respectively.

DISCUSSION

Mortality varied considerably across treatment levels. The lower treatment concentrations (44.8, 89.5, and 233.0), even with continuous no-choice feeding, failed to cause sufficient trauma to the prairie dogs to result in death, while all but 2 prairie dogs were susceptible to warfarin intoxication in the higher treatment levels (407.0 and 777.6).

The effects of the warfarin on illness of prairie dogs appear not to be dependent on treatment level of the baits. Prairie dogs in all treatment levels showed signs of intoxication by day 6 and the severity of symptoms was similar across treatment groups. Differences in severity of illness were observed later (by day 10) after the prairie dogs had begun to reduce bait consumption because of illness. As a result of the reduced bait consumption, prairie dogs within the 44.8 ppm group, began showing signs of recovery as early as day 13. Seven of the 10 prairie dogs survived in this treatment group. Also, in the other treatment groups, prairie dogs were not consuming much bait after day 10 because of illness, but these individuals did not show signs of recovery until the post-test observation period, except for 1 prairie dog in each of the 89.5 and 233.0 ppm groups. In an extreme case, prairie dog M23 lost 185 grams (21% of its initial body weight) over the 25 days of test substance exposure and post-test observation period, leaving the animal very thin, but it survived.

The higher treatment levels (407.0 and 777.6 ppm) produced illness longer, and only 2 of the prairie dogs were able to overcome the trauma of the higher doses. This suggests that with the larger dose of warfarin, a biologically active amount is present for a longer period.

Mortality is expected to be variable and physiological difference within an animal species is common (Amdur et al 1991). The results suggest physiological variation because of the large variation in reaction to the different treatment levels. The 407.0 ppm group had 100% mortality, but the higher dose, 777.6 ppm, had 80% mortality. From this group, 1 prairie dog of each sex survived. I believe these 2 individuals survived because of their physiological differences. Though the male ate less than the mean of the

treatment group, he survived a substantial amount of warfarin consumption.

Bait consumption in the control group was significantly higher than any of the treatment groups. Consumption could have been affected by the presence of warfarin, causing decreased palatability, but most likely, the treatment groups were reducing intake because of illness associated with the warfarin. Signs of warfarin poisoning appeared at a similar time (days 5-11) and then consumption decreased throughout the treatment groups because of illness (days 10-15). The control prairie dogs continued to eat throughout this period because they were healthy or showing no signs of poisoning from warfarin. Warfarin consumption was highly correlated with treatment level. Palatability likely did not affect bait consumption because ingestion of warfarin did not decrease at the higher treatment levels. Meehan (1973) reported higher warfarin concentrations (1,000 to 2,000 ppm) as being unpalatable, but in anticipation of this problem, we used a high purity of warfarin and disguised the taste with feed additives, which resulted in no palatability problems. Even at the highest dose, 777.6 ppm, neophobic response or taste aversion to the dosage were not evident.

The LC_{50} test provides an estimate of toxicity and predicts the required concentration of bait to kill 50% of the population, encompassing a range of responses of hypersusceptibility to resistance (Amdur et al 1991). The LC_{50} and LC_{90} values helped illustrate why different susceptibility to compounds can cause extreme changes in application rates. A high control of the target species is preferred, and if LC_{90} values are high, the application rates must be increased to account for resistant individuals. If the LC_{50} and LC_{90} values are similar, the population has less physiological variability.

An LC_{50} value was calculated for the actual results and then 2 alternative analyses

were performed. Little change was noticed in the LC_{50} of all analyses, but a noticeable difference was evident with the LC_{90} values. The two animals that survived the test in the 777.6 ppm treatment caused the actual LC_{90} value to more than double, compared to the alternative analyses. If 100% mortality had been achieved in the 777.6 ppm group, the LC_{90} would be one-half of the actual analysis. The expected result would be 100% mortality in the 777.6 ppm treatment, if the 407.0 ppm group had 100% mortality. Efficacy should increase as dosage increases.

Since 2 prairie dogs survived in the 777.6 ppm group, an increase in application rates of the bait should occur. Adjustments in application rates are made for such a variability in susceptibility. The apparent large range of physiological differences of the prairie dogs, therefore, require a higher dosage to achieve high mortality.

An identical pilot study (Genesis Laboratories, Inc., study #96012), except for the presence of alfalfa cubes, indicated prairie dogs as having an apparent resilience to the warfarin baits. A factor believed to be the cause of the lack of efficacy was the presence of alfalfa cubes. According to the United States-Canadian Tables of Feed Composition (Anonymous 1982), dry alfalfa can have as high as 14.2 mg of fat-soluble vitamin K/kg. Vitamin K is needed by the liver to produce prothrombin (factor II), a major component of the blood clotting mechanism (Donoco and Haft 1976, Seegars and Walz 1986). Other factors directly involved with vitamin K are the factors VII (serum prothrombin conversion acclerator), IX (plasma thromboplastin component), and X (Stuart-Prower factor). In short, these factors, along with factor II, are most important in beginning the clotting system, which is often referred to as a "clotting cascade." The positive feedbacks or this procedure continue to amplify the reaction intensity. When this cascade is

inhibited by warfarin, the result is a failure in blood coagulation (Church and Pond 1988).

With a similar study design in this study, and the removal of the alfalfa cubes, high mortality was achieved in the higher treatment groups, demonstrating diet is an important factor in the efficacy of warfarin. In addition to dietary consumption of vitamin K, other methods of vitamin K acquisition are possible. Vitamin K stored in fat can provided nourishment through vitamin deficient periods (Church and Pond 1988). Vitamin K is fat soluble and is able to be stored within fat along with vitamins A, D, and E (Church and Pond 1988, Machlin 1984). Diets deficient in fat-soluble vitamins can be consumed over a longer period of time before deficiency signs appear, than diets deficient in water-soluble vitamins (C, B_6, B_{12}, thiamin, and riboflavin). The water-soluble vitamins must be taken daily for sufficient health whereas the fat-soluble vitamins do not need to be taken as often (Church and Pond 1988, Machlin 1984).

The prairie dogs used in this study were captured in late fall, and were probably storing nutrients for the upcoming winter in the form of fat, for necropsies revealed large amounts of fat after death or test termination. Most of these animals lost weight during the study. The great weight loss of prairie dog M23 could have provided sufficient sustenance from metabolized vitamin K from the fat. The vitamin K could have been supplied from the fat to the liver for the metabolism of warfarin and reversal of the anticoagulant action. To what extent this action takes place is not known.

Vitamin K can also be replaced by gut bacteria (Hadler and Buckle 1992). Bacteria-synthesized vitamin K can be absorbed in the lower part of the intestinal tract, where the bacterial populations are greatest (Machlin 1984). Specifically, vitamin K is absorbed in the large intestine of mammals sufficiently to prevent deficiency symptoms

when presented a vitamin K-deficient diet (Hollander and Gitnick 1988). Even without the supplying of vitamin K through the diet or fat reserves, the prairie dogs still had the potential to synthesize vitamin K from gut bacteria and provide another antidote for warfarin poisoning (Hadler and Buckle 1992).

It appears that a sufficient supply of vitamin K can be acquired from diet, fat, and gut bacteria to produce an antidote in most situations. In this study, deaths increased as the warfarin concentration level increased, and death was caused by a large range of warfarin consumption. The 2 extremes of warfarin consumption, 6.3 and 352.4 mg warfarin/kg body weight, caused death in both cases, signifying physiological differences, and that more warfarin is needed to combat the effects of vitamin K if it is being accrued from extraneous sources. Metabolic or physiological differences in the prairie dog likely resulted in varying susceptibilities to warfarin.

Tissue analysis revealed a high correlation between warfarin consumption and tissue accumulation. Variation could be explained by differences in bait consumption, metabolic breakdown of warfarin, and time of warfarin exposure before death. First, prairie dogs consumed different amounts of bait which should result in different warfarin tissue residues. Second, the warfarin metabolized at different rates in the system of the prairie dogs before death and the halt of degradation from freezing of the carcass. Both factors add to differences in the residue results. Finally, if any metabolic differences are evident, this could again magnify change in the residue levels.

The analysis of warfarin bait showed a decrease in warfarin as the dose increased. Baits were initially over-formulated to compensate for loss, but apparently not enough to gain the target dosages. The warfarin concentration levels by treatment in the

concentration verification, freezer storage, and animal room sample analyses were similar indicating that the warfarin bait is very stable in the conditions encountered in the study. A low coefficient of variation (CV), *.*% shows high precision in the data. All CV values for these analyses were low, therefore, the analysis procedure was precise.

MANAGEMENT IMPLICATIONS

In this laboratory study, the 407.0 and 777.6 ppm warfarin baits were shown to be effective in the control of the black-tailed prairie dog. The lower treatment levels do produce mortality, but to an extent that survival rates are unacceptably high and that reinfestation could occur quickly.

For effective control, warfarin bait should be applied in early spring, when prairie dogs are stressed with low body weight from the over winter loss of fat reserves, and when forage is relatively low in biomass and nutritional quality.

The aforementioned characteristics of warfarin deem it safe to wildlife and humans. Antidotes for warfarin poisoning are available in the form of vitamin K in diet, fat, and gut bacteria, and for humans by injection. The reversal of the poisoning can be achieved easily if observed abruptly. For these reasons, it is environmentally safe to humans, but can be detrimental to prairie dogs targeted for control.

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Table 1. Mortality and consumption of warfarin by black-tailed prairie dogs fed 6 different concentrations of warfarin bait for 15 days.

Treatment Level (ppm)	n	Mortality (%)	Mean Warfarin Consumption (mg warfarin/kg body weight) (Range)
Control (0)	10	0	0
44.8	10	30	13.73 (6.30-21.52)
89.5	10	50	29.85 (16.27-43.31)
233.0	10	60	55.31 (15.99-107.65)
407.0	10	100	161.42 (65.08-208.16)
777.6	10	80	239.94 (128.63-352.40)

Table 2. Change in body weight[1] of black-tailed prairie dogs exposed to 6 different concentrations of warfarin bait for 15 days.

Treatment Group (ppm)	Mean Change in Weight (g)		
	Males and Females	Male	Female
Control (0)	31.2	51.4	11.0
44.8	-77.0	-111.2	-42.8
89.5	-80.7	-110.8	-50.6
233.0	-112.7	-118.2	-107.2
407.0	-94.7	-128.2	-61.2
777.6	-142.2	-170.2	-114.2

[1]Body weights were measured on Day 0 of the feeding test and at study termination or at time of death.

Table 3. Analyses with High Performance Liquid Chromatography (HPLC) of warfarin residues within the whole body tissue of the black-tailed prairie dogs.

Treatment Level (ppm)	Sample Identification[1]	Concentration (ppm)
44.8	M13	0.091
44.8	F17	0.631
44.8	F18	0.080
233.0	M32	1.495
233.0	M34	1.528
233.0	F35	0.509
777.6	M51	1.139
777.6	M52	6.072
777.6	F56	2.131

[1]M = male, F = female

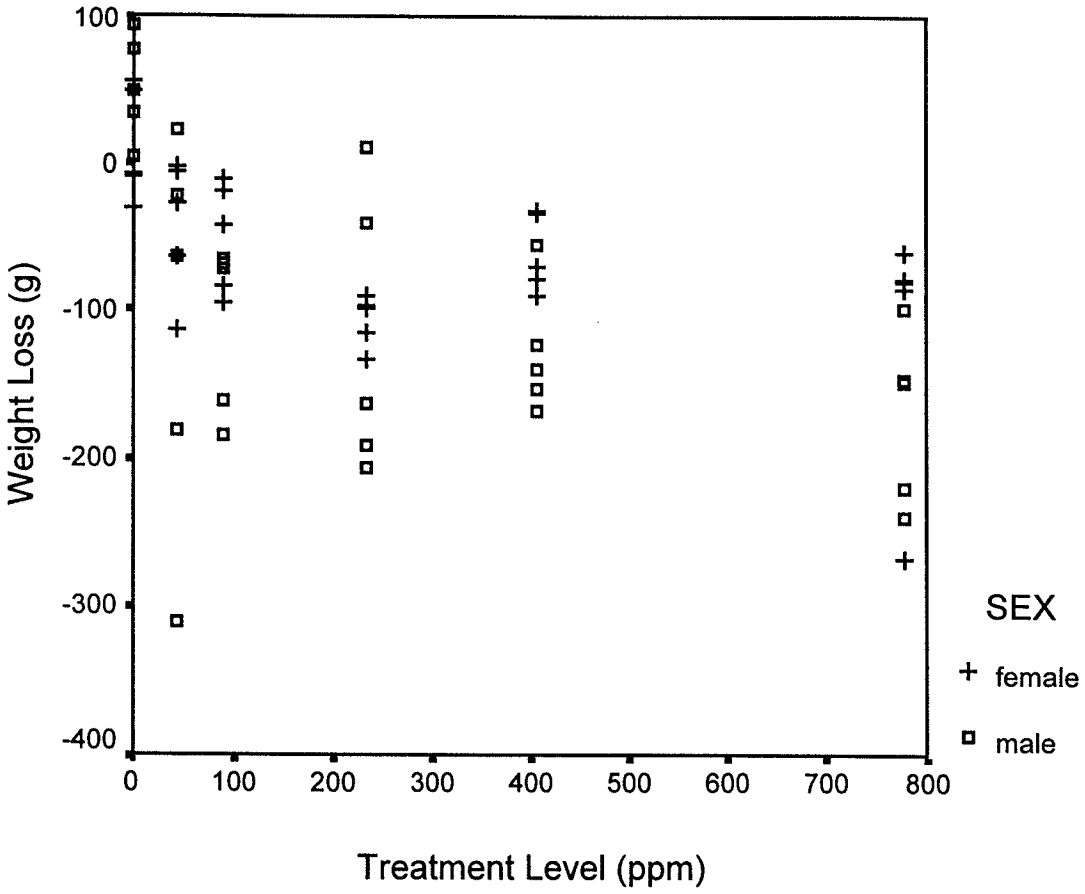

Figure 1. Black-tailed prairie dog weight loss during the exposure and post-test periods after being fed 6 different concentrations of warfarin bait for 15 days.

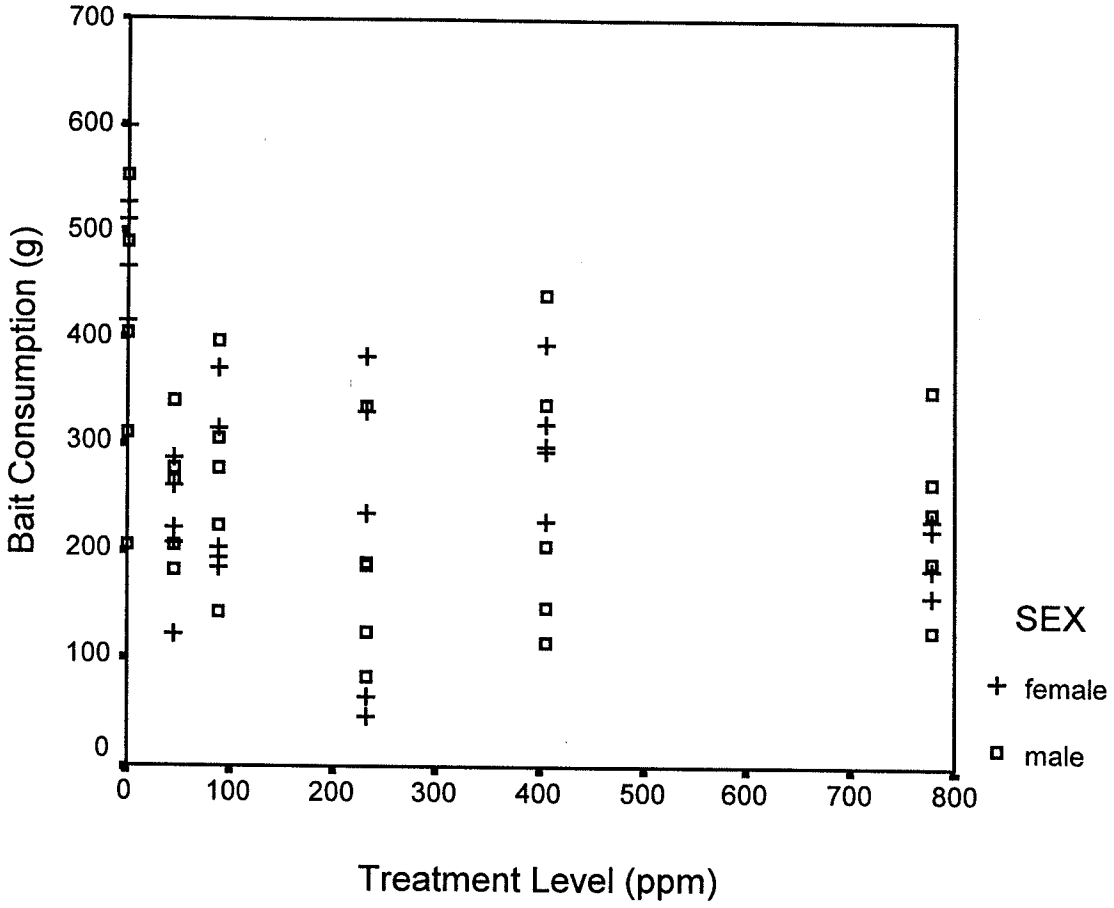

Figure 2. Total bait consumption by black-tailed prairie dogs fed 6 different concentrations of warfarin bait for 15 days.

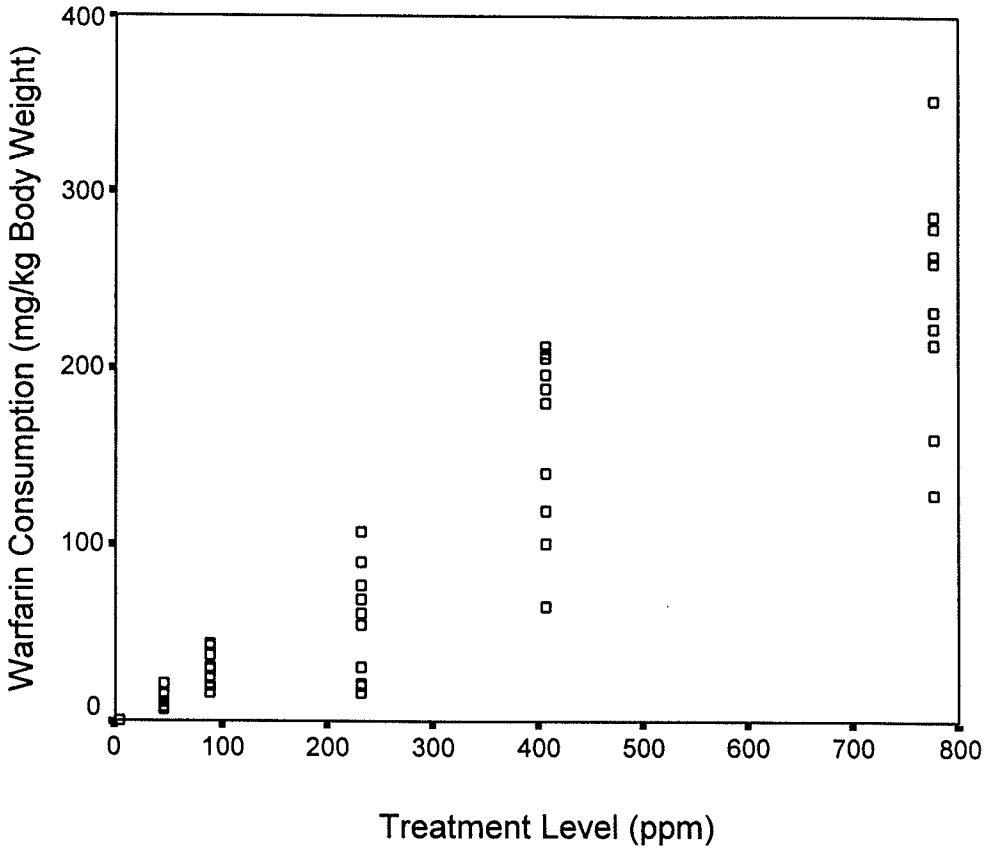

Figure 3. Consumption of warfarin by black-tailed prairie dogs fed 6 different concentrations of warfarin for 15 days.

PART III.

Secondary hazards of warfarin-fed black-tailed prairie dogs *(Cynomys ludovicianus)* to domestic ferrets *(Mustela putorius furo)*

by J. J. MACH [1]

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Summary. – Warfarin bait (500 ppm) was fed no-choice to 12 black-tailed prairie dogs (Cynomys ludovicianus) for 5 days. The prairie dogs were euthanized with CO_2 and fed no-choice to 5 male and 5 female domestic ferrets (Mustela putorius furo) for 5 days. The domestic ferrets showed no signs of warfarin intoxication after 21 days. No significant differences occurred between the male ($P = 0.380$) and female ($P = 0.143$) control ferrets and treated ferrets for prairie dog carcasses consumed and weight gain. The 500 ppm warfarin bait was determined to cause no mortality or physical harm to domestic ferrets, a surrogate model of wild mustelids, in this laboratory test situation.

INTRODUCTION

Toxicants are used to manage prairie dogs populations because they are a perceived competitor with livestock for forage resources (Taylor and Loftfield 1924, Hansen and Gold 1977, O'Meilia et al. 1982, Knowles 1986). In addition, they serve as a reservoir of disease that affect humans and other wildlife (Barnes 1982). Several toxicants have been used over the years, to poison prairie dogs, but today only 3 products are currently registered by the EPA (zinc phosphide, aluminum phosphide, and gas cartridges). Each has restrictions because of the hazards they pose to non-target wildlife and humans.

I am proposing warfarin as a possible tool, because it is relatively nontoxic to birds (Christopher et al. 1984; Hagan and Radomski 1953), it quickly degrades in the gastro-intestinal tract (half-life of 42 hours) (Ford 1993), it is rapidly excreted from the

body (Wong and Solomonraj 1980), animals often do not develop "bait shyness" (Meister 1995), and it is relatively safe with respect to secondary poisonings (Aulerich et al. 1987).

The study was designed to determine if warfarin caused secondary hazards to domestic ferrets when fed warfarin-treated black-tailed prairie dogs. The objective of the test was to determine the risk of warfarin by observing if it affects ferret feeding behavior, weight gain, and physical health.

ACKNOWLEDGEMENTS

My most sincere and deep-felt thanks go to my supervisor, and president, of Genesis Laboratories, Inc., Richard M. Poché, for providing funding, laboratory area, and equipment necessary to complete this toxicity experiment. Thanks go to the technicians of Genesis Laboratories, Inc., Scott Piotrowski, Chris Gates, Patrick Devers, Lisa Carlet, and Jeff Borchert, for their help in trapping, animal care, and analytical support during the secondary hazard laboratory studies.

METHODS

Test System - Prairie dogs

Prairie dogs were live-trapped and transported to Genesis Laboratories, Inc. and placed into individual cages for the testing period. The animals were placed into an official acclimation in which environment was monitored and observations of health were recorded for 10 days. During the exposure period, the prairie dogs were fed 500 ppm warfarin bait *ad libitum* for 5 days, and then a 2-day post-test observation period. The animals were observed twice daily during these 2 periods. The prairie dogs were

euthanized with CO_2 at the end of the post-test observation. The animals were immediately wrapped and a labeled plastic bag and frozen until the ferret exposure portion of the study. Details of the methods and results of the preparation of the prairie dogs for the ferret secondary hazard test are explained in Appendix 2.

Test System - Ferrets

Domestic ferrets were selected as test animals because they are phenotypically indistinguishable from wild ferrets of Europe (Mustela putorius). Twelve domestic ferrets, 16-19 weeks old, (6 males and 6 females) were transported to Genesis Laboratories, Inc.; Fort Collins, Colorado from Marshall Farms, USA Research Division of North Rose, N. Y. by air freight. The ferrets were were dusted with Adams™ Flea and Tick Dust II (0.5% pyrethrin, SmithKline Beecham Animal Health, West Chester, PA), to control ectoparasites. Five males and 5 females were randomly selected for the treatment group using computer program "RAN30." One male and 1 female were used for the control group.

Each ferret was held in an individual plastic coated wire screen bottom cage. Cages had a surface area of 4650 cm^2 and a height of 46cm (National Research Council 1992). Ferrets received a basal diet of Ultra Blend Ferret Diet (8 in 1 Pet Products, Inc., Hauppauge, New York) and water *ad libitum*. Bedding was changed and cleaned weekly, while the water and feed were checked daily. Cages and racks were not cleaned during the study because of the mode of action of the test substance. Handling of the animals could cause lesions, bruises, or injury, that could bias mortality estimates or calculations (Penumarthy and Oehme 1978).

The 12 ferrets were acclimated to the cages, animals room, and basal diets for 7 days. The ferrets were observed daily during the acclimation period for general health and abnormalties. All ferrets were inspected by a veterinarian to assess their suitability for testing. We measured the body weight of each ferret at the initiation and termination of the acclimation period. Ferret rooms were maintained between 14 and 27°C, 10 and 80% relative humidity, and a 12 hours light, 12 hours dark lighting regime. Ferrets were weighed by placing each one in a cloth bag and then into a plastic container on a Ohaus balance. Body weights were recorded at the initiation of acclimation, day 0, day 5, and day 26 (test termination).

The ferrets were pre-conditioned to eating whole prairie dog carcasses during a 3-day period after the acclimation period. Twelve (6 males and 6 females) untreated prairie dogs were sacrificed by asphyxiation with CO_2 and presented to the ferrets immediately no-choice. Each prairie dog carcass was weighed at presentation and at the end of the exposure period. Four of the 12 ferrets, females, had not begun to eat after 1 day of exposure to the untreated carcasses. The ferrets had never been exposed to eating raw meat in carcass form, so the skin was cut from the jaw bone to mid-sternum in an effort to entice the ferrets to eat.

Since female ferret F11 only consumed 24 grams of prairie dog tissue over the 3-day period, the ferret was not used in the treatment group. F11 was placed into the control group and switched with ferret F2, which was a regularly eating prairie dog. F2 had eaten a similar amount of carcass compared to the other females within the test.

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Toxicity Test

Immediately following the 3-day conditioning period, a 5-day toxicity test was initiated. On day 0 of the toxicity test, each of the 10 ferrets in the treatment group was provided one thawed, warfarin-fed prairie dog carcass no-choice. The weight of each carcass was recorded before presentation to the ferrets. Observations of tissues consumed by the ferrets were recorded daily. A new carcass was presented only if a ferret consumed a majority of the carcass. To ensure that the ferret ate most of the tissues and organs throughout the body that could contain warfarin and/or its metabolites. Remaining portions of each carcass were collected and weighed to determine consumption rates. The same procedures were followed for the 2 ferrets in the control group except that they were presented with prairie dogs that were not exposed to the warfarin.

The prairie dog carcass that was presented to ferret F9 was incised from the jaw bone to the mid-sternum to entice eating. This was one of the ferrets that was enticed into eating during the pre-conditioning phase. After no obvious feeding had occurred from the control ferret F11, 15 grams of ferret feed were placed in the incision to entice the ferret to eat the carcass.

The ferrets were observed twice daily during the feeding and post-test observation periods to monitor their health. Observations were to include physical and behavioral signs that could lead to the identification of anticoagulant poisoning. Observations were performed at these time to limit any undue stress to the animals which could induce mortality from frequent handling or human visits into the test room (Penumarthy and Oehme 1978).

Total consumption of warfarin by the ferrets was calculated by using the total

amount of bait consumed by the prairie dogs. An assumption was made that the warfarin had not degraded within the prairie dog system since intake. The bait consumed by the prairie dog was multiplied by the concentration of the bait to estimate the amount of warfarin consumed, and then divided by the weight of the prairie dog to get mg warfarin/kg body weight of prairie dog. This figure was then multiplied by the amount of prairie dog consumed by the ferret in kilograms. Assuming equal distribution of warfarin within the system of the prairie dog, this procedure provides the amount of warfarin consumed by a ferret in milligrams, with zero degradation.

The post-treatment observation period lasted 21 days. This period was sufficient to notice any effects to the ferrets, for the half-life of warfarin within the body is 42 hours (Ford 1993). No analysis of ferret tissues was performed because the ferrets failed to show any signs of warfarin poisoning throughout the 26-day period.

Data analysis was performed using SPSS for Windows, release 7.5 (November 14, 1996, Standard Version, Copyright © SPSS Inc. 1989-1996). A linear regression was used to compare relationships between dependent (mortality, weight loss, and consumption) and independent variables (treatment level). T-tests were performed to identify sex-specific differences among treatment animals.

RESULTS

Pre-Conditioning

Feeding behavior of the ferrets was consistent among treatment animals. They would consume parts of the head and neck, then slowly consume the parts of the thoracic region and forelegs. By the third day, they would move to the abdominal region and eat

parts of the organs. Four of the females had not eaten any of the prairie dog carcasses on day 1, so the carcasses were incised to facilitate consumption. This method helped 3 of 4 female ferrets begin to feed. The ferrets consumed bones, organs, and muscle tissue. They failed to eat the skull, vertebrae, and skin in the pre-conditioning period.

Toxicity Test

No ferrets died during the test and post-test observation periods. The treatment and control ferrets completed the test with absolutely no signs of toxicosis. The mean body weights of ferrets used in the control group and treatment group were 1,394 and 1335 g, for the males, respectively, and 616 and 751 g, for the females, respectively. The mean body weights of ferrets at day 21 used in the control group and treatment group were 1670 and 1664 g for the males, respectively, and 826 and 828 g, respectively. The percent mean body weight gain during the toxicity test by the control group for the males and females was 16.5 and 25.4%, respectively. The percent mean body weight gain during the toxicity test by the treatment group for the males and females was 19.7 and 9.3%, respectively.

Figure 1 shows the rate at which Marshall Farms' male and female ferrets grow to maturity (Marshall pers. comm.). The ferrets used for this test were purchased from Marshall Farms at an age of 14 weeks old. Figure 2 shows the rate of growth for the domestic male and female ferrets, treated and control, during the acclimation, exposure, and post-test observation periods. Figure 2, in comparison to Figure 1, shows the rate of growth of the Genesis test ferrets to the ferrets of Marshall Farms, which are raised to be healthy. The weights of the Genesis test ferrets are very similar to weights of the

Marshall Farms ferrets. The 8:1 Ultra Blend Ferret Diet, fed before and after the carcass presentation, is very similar to the diet used for the Marshall Farms ferrets (Marshall pers. comm.).

The rates of gain for the male ferrets was strongly correlated to the amount of prairie dog consumed ($r = 0.919$), but there is not a strong correlation with the female rates of gain and the amount of prairie dog consumed ($r = 0.339$).

All of the ferrets showed weight gains between 335 to 656 g for the males, and 82 to 215 g for the females. The two control ferrets gained 486 g and 156 g respectively. These 2 weight values of the control ferrets are almost the calculated means of the weight gains for the treatment males and females. Mean carcass consumption during the 5-day exposure period, by the control and treatment groups was 564 and 559 g/kg body weight, respectively (Table 3). Mean carcass consumption by treatment male ferrets was 777 g/kg body weight, and the treatment females consumed 341 g/kg body weight. The male and female control ferrets consumed 651 and 476g, respectively. The 5 male ferrets had a mean warfarin ingestion of 29.0 mg/kg body weight. The 5 female ferrets had a mean ingestion of warfarin of 21.1 mg warfarin/kg body weight. The warfarin values are based on the prairie dog consumption of warfarin bait without any degradation within the prairie dogs' system.

The male control ferret was not significantly different in its consumption of prairie dog ($\underline{P} = 0.380$) or the gain in body weight ($\underline{P} = 0.380$) compared to the male treatment ferrets. Also, the female control ferret was not significantly different in its consumption of prairie dog ($\underline{P} = 0.143$) or the gain in body weight ($\underline{P} = 0.380$) compared to the female treatment ferrets.

Mean carcass consumption during the 5-day exposure period, by the control and treatment groups was 563.5 and 558.7 g/kg body weight, respectively (Table 3). Mean carcass consumption by treatment male ferrets was 777 g/kg body weight, and the treatment females consumed 341 g/kg body weight. The male and female control ferrets consumed 651 and 476g, respectively. The 5 male ferrets had a mean warfarin ingestion of 29.0 mg/kg body weight. The 5 female ferrets had a mean ingestion of warfarin of 21.1 mg warfarin/kg body weight. The warfarin values are based on the prairie dog consumption of warfarin bait without any degradation within the prairie dogs' system.

Throughout the exposure period, the male ferrets continued to eat the treated prairie dog carcasses without any enticing. In order of consumption, they ate the thoracic region, head, neck, and then consumed tissue moving backwards to the tail; eating all but, skin, large bones and tail. The females followed a similar pattern, but started eating the head and neck first, then working to the posterior end of the body. Female ferrets F9 and F11 started 1 and 2 days after the other females, but they quickly ate as much as the other females by the end of the test.

On day 4, 3 male ferrets were given a second prairie dog, and they began eating in the same pattern as they had before. The female ferrets ate slower, and as a result, much more carcass was remaining, mostly bones, skin, and organs of the abdomen. On the final day of exposure, all carcasses were removed and the ferrets were given the 8:1 Ultra Blend Ferret Diet *ad libitum*.

Bait analysis

The actual warfarin bait concentration was 480.0 ± 31.0 ppm with a coefficient of

variation (CV) of 6.45%. The bait was 96.1% of the nominal concentration (500 ppm). Very little warfarin was lost during the analysis process.

DISCUSSION

Pre-conditioning and Toxicity Test

No physical signs of toxicosis were observed during any part of the testing period, and the ferrets appeared to remain healthy, for no negative physical or behavioral signs were observed from the warfarin. Warfarin appeared to have little effect on the weight gain of the ferrets. The treatment male and female ferrets ingested an estimated mean of 29 and 21 mg warfarin, respectively, through the treated prairie dogs during the 5-day test period. Consumption rates of prairie dogs and rates of gain among the males and females were similar across the control and treatment groups, suggesting warfarin had little effect on the ferrets. The female ferrets showed more variability between consumption of prairie dogs and the amount of body weight gain, unlike the males.

The male ferrets' consumption of warfarin was highly correlated to amount of warfarin within the prairie dog. If the ferrets would have been affected by the warfarin, they would have decreased their consumption of prairie dog as the concentration of warfarin increased. A different relation was evident with the females. They exhibited a relation suggesting they were encountering harmful effects of the warfarin secondarily. In conjunction with their feeding habits and the randomization of the prairie dogs, they displayed a wide array of consumption of the prairie dogs. Also, during the course of the pre-conditioning and exposure period, it was difficult to entice the female ferrets to eat raw meat. In general, the female consumption is sporadic. I suggest the females showed

no change in normal feeding behavior because of the warfarin-treated prairie dogs, but this was caused because of the presence of an entirely new food item.

Further, the similar weight gains of the Genesis ferrets on test, and the ferrets of Marshall Farms showed that the ferrets were healthy even after the consumption of the warfarin. Discrepancies were evident, but there was a small sample size ($n = 6$) of Genesis ferrets which had to acclimate to a new environment with minor stresses. The change in environment could have attributed to the differences in the body weights compared to the Marshall ferrets.

Differences in feeding habits were observed between the pre-conditioning and the exposure period. During the pre-conditioning period, the male ferrets began feeding on the neck and head region first, but during the exposure period, the male ferrets began feeding upon the internal organs of the thorax first. Apparently, a preference to the internal organs for taste or nutrient content exsists. Gad and Chengelis (1988) report ferrets eating to the limit of their caloric needs and halting feeding until food is again needed, and that ferrets pass fiber through their system in 3 to 4 hours. With such a rapid charge through the system, it would appear that some of the warfarin would exit with the feces.

After the ferrets became experienced with eating raw meat in the pre-conditioning period, the males developed an affinity for the thoracic organs, for they consumed this part of the prairie dog carcass first. Warfarin is known to be concentrated in the liver, but it also has measurable amounts in the adrenal glands, lungs, bone marrow, kidneys, and lymph nodes (Machlin 1984). The ferrets showed no signs of intoxication, even though they ate some of the more consequential organs, which have the ability to accumulate the

toxin. An attribute of warfarin is its ability to quickly degrade within the gastro-intestinal tract of the prairie dog with a half-life of 42 hours (Church and Pond 1988), compared to a more toxic anticoagulant, chlorophacinone, which has a half-life of 120 days in dogs (Lipton and Klass 1984), and can involve complicated treatments of antidotal vitamin K for more than 45 days (Jones, Growe, and Naiman 1971). One human case involved vitamin K treatments for 132 days after ingestion of chlorophacinone (Burucoa et al 1989).

A "worst case scenario" developed because of the prairie dog exposure to the bait and the eating style of the ferrets. To illustrate, the prairie dogs were euthanized early in post-treatment, which would result in high concentrations of warfarin remaining in the gastro-intestinal system, as opposed to waiting for a longer post-treatment period that would drop the amount of warfarin within the system. Also, the male ferrets preference to eating the thoracic organs and liver first, the organs that concentrate the warfarin, elevated the risk of warfarin intoxication to the ferrets. A similar action also occurred with the female ferrets to a lesser extent. With the higher amount of warfarin given to the ferrets and at a quicker rate, the ferrets still showed no signs of intoxication.

Bait analysis

The analytical bait method offers a reliable, reproducible, and rapid analysis of the test substance due to the solubility of surface applied warfarin in methanol. The bait appeared to be slightly lower than the target concentration, but this was accounted for in any calculations. In a companion study, the same warfarin bait was shown to degrade little in freezer storage conditions exhibiting the stability of the compound.

CONCLUSIONS

Warfarin-contaminated prairie dog carcasses were presented to 10 physically and behaviorally healthy ferrets (5 males and 5 females) in a no-choice feeding regime for 5 days. Following the 5-day no-choice feeding, the ferrets were maintained for 21 days with 8:1 Ultra Blend Ferret Diet. No ferrets were found dead or having visible signs of anticoagulant intoxication due to warfarin poisoning. Furthermore, analyses of prairie dog carcass consumption and ferret weight gain showed no significant differences between the control ferrets and the treated ferrets. The 500 ppm warfarin bait was determined to cause no mortality or physical harm to domestic ferrets, a surrogate model of wild mustelids, in this laboratory test situation.

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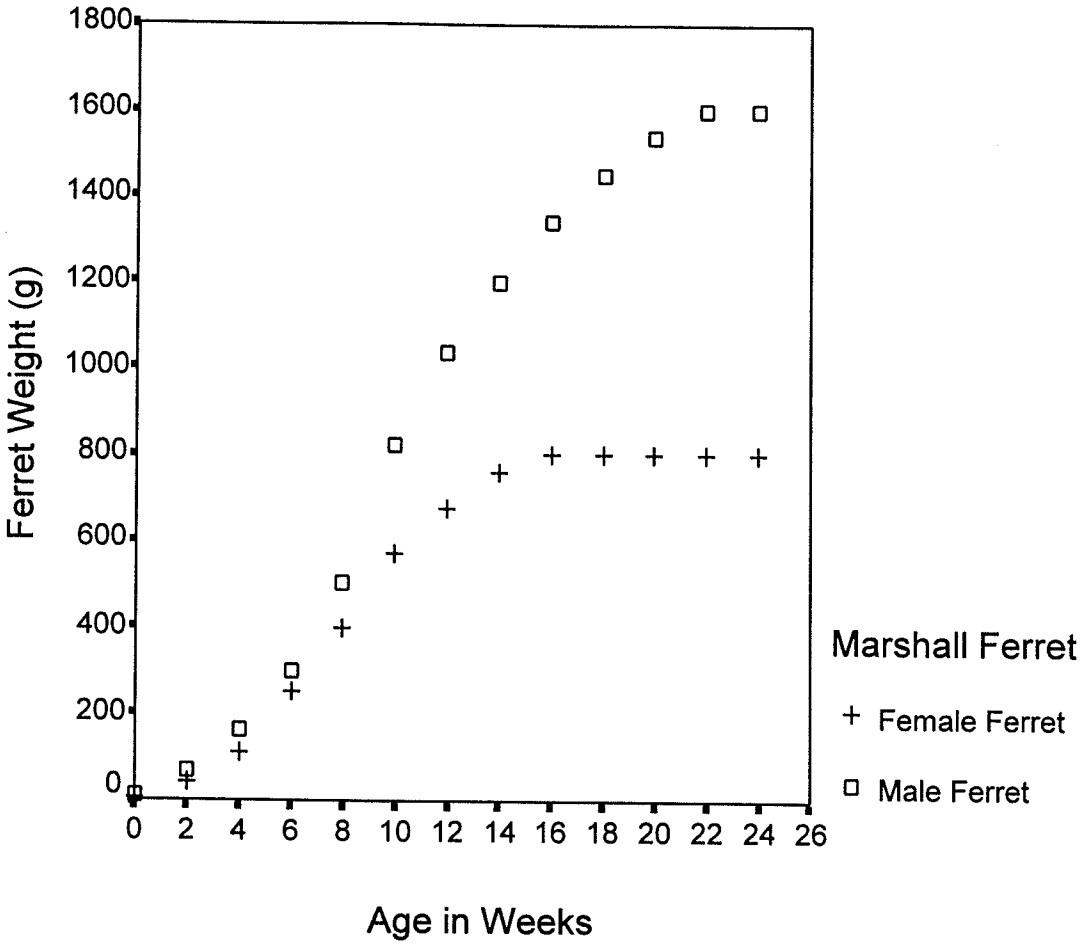

Figure 1. Weight gain of Marshall Farm male and female domestic ferrets from birth to maturity. (Marshall pers. Comm.).

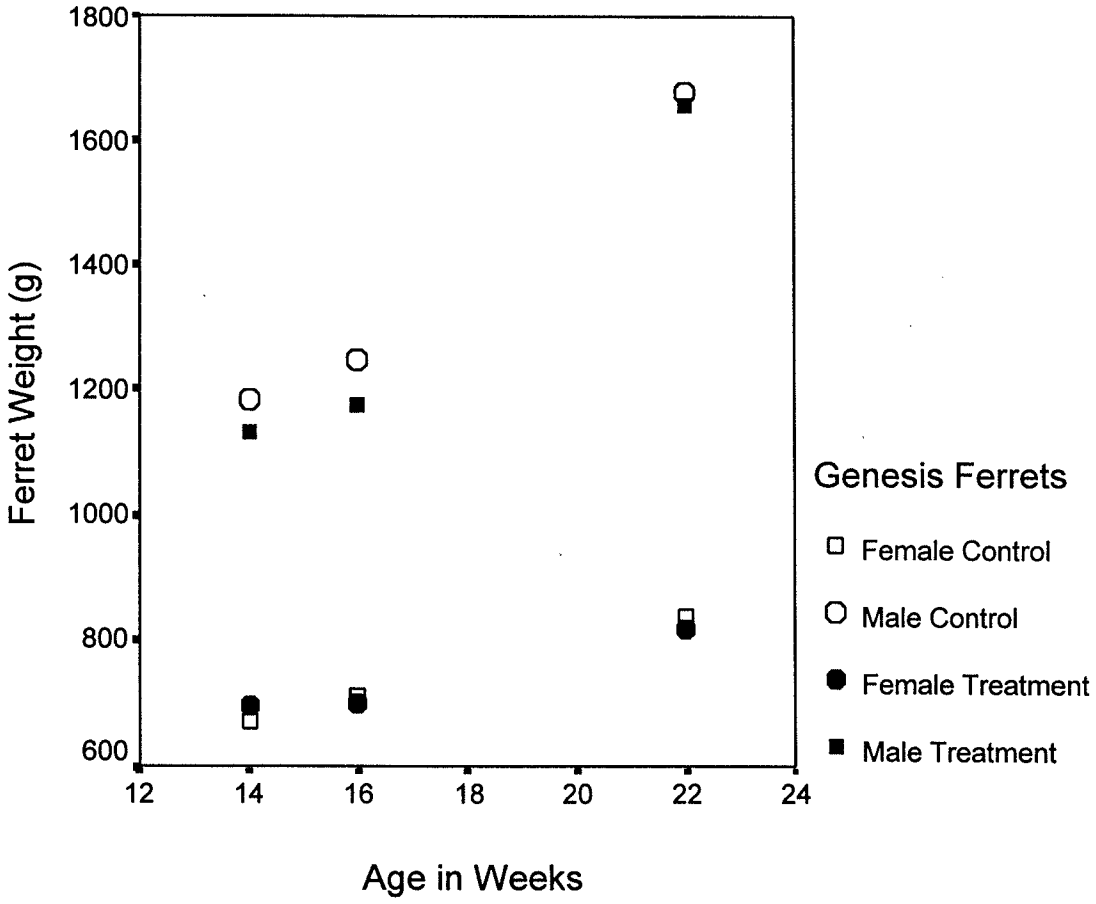

Figure 2. Weight gain of male and female domestic ferrets during the acclimation, exposure, and post-test observation periods.

Table 1. Consumption of warfarin-fed prairie dog carcass consumed by domestic ferrets during the 5-day exposure period.

Number	Sex	Body Weight (g)[1]	Carcass Consumption (g)	mg warfarin / kg Body Weight
Control				
1	M	1394	651	0
11	F	616	476	0
Treatment				
3	M	1264	889	37.9
4	M	1396	834	35.9
5	M	1268	428	5.7
6	M	1403	862	20.6
7	M	1344	871	45.0
Mean		1335	777	29.0
8	F	735	386	10.2
9	F	761	392	25.6
10	F	782	294	26.1
2	F	720	288	19.3
12	F	759	343	24.4
Mean		751	341	21.1

[1]Body Weight at Day 0

APPENDIX I

ANALYTICAL METHODS

METHODS AND MATERIALS - BAIT

A high performance liquid chromatography (HPLC) method was used to determine the concentration of warfarin in the test substances. The adapted method employed a reversed phase column and ultraviolet detection.

Chromatographic System

The chromatographic system consisted of a Waters model 501 high pressure pump, a Rainin Dynamax AI-2 autosampler with a Rheodyne 6-port air-actuated injector, and a 20 µl sample loop. To separate of all impurities from the active ingredients, we used an octadecylsilane column (3.9 mm x 300 mm, 10 µm particle size, µBondapak C18, Waters, Inc., Milford, Massachusetts) with an octadecylsilane guard column (4.6 mm X 10 mm, 10 µm particle size, Econosil C18, Alltech, Inc., Deerfield, Illinois) at room temperature (25°C). An isocratic mobile phase consisting of 60% acetonitrile : 30% water with pH adjusted to 3.0 using phosphoric acid and potassium hydroxide pellets was mixed, filtered, and degassed by vacuum through a 0.45 µm nylon filter membrane prior to introduction into the system. The flow of the pump was maintained at 1.5 ml/min using a Waters model 680 Automated Gradient Controller.

A Waters model 484 tunable absorbance detector was used to monitor the column effluent. The wavelength was held constant at 286 nm for all samples and standards in this study. The integration was performed by a Waters model 745B data module.

The HPLC integration parameters of Table 1, which set the HPLC to do specific functions, were maintained throughout the bait analysis.

HPLC Mobile Phase Preparation

Phosphoric acid (1.85 ml) was added to 800 ml HPLC-grade water and adjusted the pH to 3.0 (by pH paper) using potassium hydroxide pellets. HPLC-grade acetonitrile (1200 ml) (60% v/v) was added to the aqueous solution, vacuum filtered through a 0.45 μm filter, and placed into a clean bottle.

Analytical Standards

Warfarin technical (Lot # 21H0477, Sigma Chemical Company, St. Louis, Missouri, purity 99%, expiration date 3/2000) was used for the preparation of standard solutions for this study.

The warfarin stock solution was prepared from 26.2 mg of 99% warfarin diluted in 50.0 ml methanol. For the working standards, 0.5 ml of the stock solution was diluted in 100 ml methanol for a final concentration of 2.59 μg/ml, 1.0 ml of stock solution was diluted in 100 ml methanol for a final concentration of 5.19 μg/ml, 2.0 ml of stock solution diluted in 100 ml methanol for a final concentration of 10.38 μg/ml, and 5.0 ml of stock solution diluted in 100 ml of methanol for a final concentration of 25.9 μg/ml. Four milliliters of internal standard (described later) was added to each dilution prior to dilution with methanol. All volumetric measurements were made with class A glassware. An aliquot of each standard was filtered through a 0.2 μm (PTFE) syringe filter into an autosampler vial for HPLC analysis.

Internal Standard Preparation

4-chlorobenzophenone (Lot #04514LF, Alldrich, Inc., Milwaukee, Wisconsin, expiration date 3/2000) was used for the preparation of internal standard solutions for this study. An internal standard stock solution was prepared from 58.4 mg of 4-chlorobenzophenone, placed in a 100 ml class A volumetric flask and diluted to volume with methanol. The solution was sonicated for 1 minute to aid in dissolving the 4-chlorobenzophenone.

Sample Preparation for Concentration Verification

Six samples of bait were collected from each treatment level. Each sample was individually ground in a Cyclone Sample Mill (Udy Corporation, Ft. Collins, Colorado) and a 2.00 ± 0.10 g of test substance was place into a tared, labeled, 50 ml centrifuge tube. Approximately 22 ml of methanol was added by graduated cylinder and the samples sonicated at 40°C for 5 minutes manually shaking the samples every minute. The samples were then centrifuged for 5 minutes, at 6000 rpm. Samples from concentration verification levels 50 through 500 ppm were then decanted into labeled 50 ml, class A volumetric flasks. Samples from the 1000 ppm bait were decanted into labeled 100 ml class A volumetric flasks. A double extraction was performed by adding an additional 22 ml methanol, sonicating, and shaking for 5 minutes at 40°C and centrifuging for 5 minutes at 6000 rpm. The extracts were combined in respectively labeled volumetric flasks. A 2.0 ml sample of internal standard was then added with a 2.0 class A pipette and the samples were diluted to volume with methanol. An aliquot of

each sample was then taken and filtered through a 0.2 μm PTFE syringe filter into an autosampler vial for HPLC analysis.

Sample preparation for freezer and room stability samples

The sample preparation for the stability evaluation was the same as the sample preparation for the concentration verification analysis except 1 sample of each bait level was analyzed.

Analytical Method Validation, Bait Method

The bait methods used in this study have been validated according to Genesis Standard Operating Procedures. The parameters of coefficient of variation, linearity, sensitivity, reproducibility, and recovery (bias) have been determined and have met the criteria established in the SOP. The coefficient of variation (CV) was determined by comparing 6 separate sample preparations to 1 linear regression relationship. The mean of these 6 sample concentrations and the associated standard deviation was calculated. The CV was represented as the standard deviation divided by the mean and was reported as a percentage. The CV for this method was 2.68% ($n = 6$).

The linearity was determined by injecting a reagent blank and at least 3 concentrations of an analytical standard. Three separate sets were injected. This procedure was used to generate a linear regression relationship of warfarin concentration versus warfarin peak area or internal standard area. Linearity was expressed as the R^2 value for the relationship, where a perfectly straight line has a value of 1.000. The mean R^2 value of this method was 0.9997 ($n = 3$).

The sensitivity was determined by injecting the lowest analytical standard 5 times. The mean of these 5 areas produced by the HPLC and the associated standard deviation (s) was calculated. The CV was represented as the standard deviation divided by the mean and was reported as a percentage. The limit of detection was 3 standard deviations. A linear regression relationship was used to obtain a concentration. The limit of detection for this method was 0.518 μg/ml.

The analytical reproducibility was determined by injecting an analytical standard 6 times. The mean of these 6 peak areas and the associated standard deviations were calculated. The CV was represented as the standard deviation divided by the mean and was reported as a percentage. The reproducibility of this method was 1.34% (n = 6).

The recovery was determined by fortifying at least 3 samples and at least 3 blanks with a known amount of analytical standard. The recovery was calculated by dividing the actual concentration by the theoretical concentration. It was expressed as a percentage. The fortified sample recovery for this method was 97.2 ± 2.1%. The fortified blank recovery for this method was 95.5 ± 0.5%.

METHODS AND MATERIALS - TISSUE

An adaptation of the HPLC method described by Chalermchaikit (1993) was used to determine the concentration of warfarin in the test model. The adapted method employed Solid-phase extraction (SPE), a reversed phase column, and UV detection.

Chromatographic System

The chromatographic system for the tissue analyses was similar to the analyses for the

bait, except for the following parameters: chart speed setting, 1.0 cm/minute; attenuation setting, 32; end run time, 15 minutes.

HPLC Mobile Phase Preparation

The mobile phase for the tissue analysis is the same as the mobile phase for the bait analysis.

Analytical Standard Preparation

Warfarin technical (Lot # 21H0477, Sigma Chemical Company, St. Louis, Missouri, purity 99%, expiration date 3/2000) was used for the preparation of standard solutions for this study. The warfarin stock solution was prepared from 12.5 mg of 99% warfarin diluted in 50 ml methanol in a class A volumetric flask. For the working standards, 0.5 ml of stock solution was diluted in 100 ml methanol for a final concentration of 1.24 µg/ml, 1.0 ml of stock solution was diluted in 100 ml methanol for a final concentration of 2.48 µg/ml, 2.0 ml of stock solution was diluted in 100 ml methanol for a final concentration of 4.96 µg/ml, 4.0 ml of stock solution was diluted in 100 ml of methanol for a final concentration of 9.92 µg/ml, and 5.0 ml of the 9.92 µg/ml standard was diluted in 100 ml methanol for a final concentration of 0.50 µg/ml. A sample of methanol was also used as a standard blank. All volumetric measurements were made with class A glassware. An aliquot of each standard was filtered through a 0.2 µm PTFE syringe filter into an autosampler vial for HPLC analysis.

Sample Preparation

Each frozen prairie dog was thawed individually in warm water for 3-4 hours in a plastic bag. They were cut into 8 equal segments and placed into a Waring Commercial blender (Waring Products Division, New Hartford, Connecticut). An equal amount of histological grade acetone (volume of acetone : weight of prairie dog) was added to the blender to facilitate grinding and to extract the warfarin. For calculations, the weight was adjusted to account for the presence of acetone by dividing the sample weight by 1.788 (the specific gravity of acetone + 1). The carcass was blended until homogenized and placed into a freezer bag. Three 5.00 ± 0.1 g samples were taken from the mixture and placed into labeled 50 ml centrifuge tubes and either frozen or processed immediately. This sample weight accounts for the 1:1 (volume of acetone : weight of prairie dog) ratio. Samples were diluted with 15 ml of HPLC-grade acetonitrile and minced in a Tissumizer (Tekmar Corporation, Cincinnati, Ohio) for 1 minute at 60% of full speed. The samples were centrifuged for 5 minutes at 7000 rpm in an Adams Dynac centrifuge (Clay Adams Company, Parsippany, New Jersey). The samples were decanted into a clean, labeled centrifuge tube. A double extraction was performed by adding 10 ml of HPLC grade acetonitrile, mincing in a Tissumizer for 1 minute at 60% and centrifuging for 5 minutes at 7000 rpm. The extracts were combined in a class A 50 ml volumetric flask for applications to Sep-Pak filtration into HPLC autosampler vials.

Solid Phase Extraction

Approximately 4.0 g of Column Chromatography-grade alumina and approximately 10.0 ml methanol were swirled to create a slurry. The slurry was poured

into a 10 ml vertically clamped syringe (plunger removed) with an attached Silica Sep-Pak (Waters, Inc., Milford, Massachusetts). The apparatus was tapped to settle the alumina. A suction apparatus was used to draw the methanol through the Sep-Pak. In addition, approximately 4.0 ml HPLC-grade water and approximately 4.0 ml HPLC acetonitrile were separately suctioned through the Sep-Pak. A clean, labeled 250 ml round bottom flask was attached to the Sep-Pak. The sample was then incrementally poured into the syringe and suctioned through the alumina and Sep-Pak into the round bottom flask. The sample was followed by a 5.0 ml wash of acetonitrile collected in the round bottom flask as well. The round bottom flask was attached to a Buchi Rotovapor (Brinkmann Instruments, Inc., Westbury, N. Y.) with water bath at 55°C and the liquid evaporated to dryness. Once the liquid was evaporated, the flask was cooled to room temperature and 4.0 ml of warfarin mobile phase was added using a 2.0 ml class A pipette (2X) to dissolve the sample. Samples were filtered through a 0.2 μm PTFE syringe filter into an autosampler vial for HPLC analysis.

Analytical Method Validation, Tissue Method

The tissue method used in this study was similar to the bait method except for the following parameters: the CV was determined by a comparison of 3 tissue samples obtained from the same carcass, and the CV for this method was 16.11% ($n = 3$).

The linearity was determined by injection of at least 3 concentrations of analytical standards plus reagent blank. This procedure was used to generate a linear regression relationship of warfarin concentration versus the warfarin peak area. Linearity was expressed as the R^2 value for the relationship, where a perfectly straight line has a value

of 1.000. The mean R^2 value of this method was 0.9998 ($n = 3$).

The sensitivity was determined by obtaining the standard deviation (s) of replicate injections of the lowest analytical standard. The limit of detection was 3 standard deviations and this was used in a linear regression relationship to obtain a concentration. The limit of detection for this method was 0.099 µg/ml.

The analytical reproducibility was determined by obtaining the standard deviation (s) and CV of replicate injections of an analytical standard. The reproducibility of this method was 2.30% ($n = 6$).

The recovery was determined by fortifying samples and blanks with a known amount of analytical standard. The recovery was calculated by dividing the actual concentration by the theoretical concentration. It was expressed as a percentage. The fortified sample recovery for this method was 94.2 ± 25.7%. The fortified blank recovery for this method was 91.5 ± 11.7%. Table 2 lists the reagents used for the bait and tissue analyses.

RESULTS AND DISCUSSION - BAIT

The analytical bait concentration verification assays identified that warfarin concentrations were actually 12 to 25% lower than the target treatment level. The CVs were homogenous for the bait analyses. Tables 3, 4, and 5 identify warfarin concentrations in the bait of each treatment group for concentration verification, freezer storage stability, and animal room stability. Notice how the results in each of these tests of each treatment level is consistent with the other respective concentrations. The results in comparison to each type of analysis and treatment level, are relatively close, suggesting

that the bait is stable in the environment encountered during the course of the study. This bait method offers a reliable, reproducible, and rapid analysis of the test substance due to the solubility of surface applied warfarin in methanol.

RESULTS AND DISCUSSION - TISSUE

Table 6 shows how the concentration of warfarin within the prairie dog tissues increases as the treatment level increases. The CVs were homogenous for the tissue analysis, though they were higher than . The results in comparison to each treament level show an increase in concentration within the tissues as the treatment level increases ($r = 0.709$). The variation in concentration in the tissues increased as the treatment level increased, suggesting a greater difference in metabolism of the higher concentrations of warfarin bait.

The tissue method offers a reliable, reproducible, and rapid analysis of test substance in tissue. The high CV values are due to the difficult tissue matrix of whole prairie dog.

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Table 1. HPLC Bait Analysis Integration Parameters

Parameter	Time	Setting
Peak Markers	0.01	1
Peak Threshold	0.02	250
Backward Horizontal	0.03	1
Auto Zero	0.30	1
Chart Speed	0.31	0.5 cm/min
Attenuation	0.32	64
End Run	12 minutes	1
Sensitivity	Initial	1.00

Table 2. Reagents. The following reagents were used for the bait and tissue analyses.

Reagent	Grade	Lot Number	Manufacturer
Acetone	Histological	963595-36	Fisher
Acetonitrile	HPLC	967779	Fisher
		96595	Fisher
Alumina	Column Chromatography	45H3505	Sigma
Methanol	HPLC	963953	Fisher
Phosphoric acid	ACS	06730PX	Aldrich
Potassium Hydroxide	ACS (pellets)	9264770	Fisher
Water	HPLC	960297	Fisher
		966020	Fisher

Table 3. Concentration of warfarin in the 5 warfarin baits fed to black-tailed prairie dogs for 15 days.

Nominal Treatment Level (ppm)	Coefficient of Variation (CV)	Concentration (ppm)
50	8.26%	44.8 ± 3.7
100	13.18%	89.5 ± 11.8
250	7.81%	233.0 ± 18.2
500	8.55%	407.0 ± 34.8
1000	15.66%	777.6 ± 121.8

Table 4. Concentration of warfarin storage stability samples in the 5 warfarin baits for black-tailed prairie dogs.

Nominal Treatment Level (ppm)	Concentration (ppm)
50	51.6
100	83.8
250	224.5
500	400.5
1000	780.9

Table 5. Concentration of warfarin animals room stability samples of the 5 warfarin baits for black-tailed prairie dogs.

Nominal Treatment Level (ppm)	Concentration (ppm)
50	44.1
100	74.7
250	223.8
500	445.9
1000	779.7

Table 6. Analyses with High Performance Liquid Chromatography (HPLC) of warfarin residues within the whole body tissue of the black-tailed prairie dogs.

Treatment Level (ppm)	Sample Identification[1]	Concentration (ppm)
44.8	M13	0.091
44.8	F17	0.631
44.8	F18	0.080
233.0	M32	1.495
233.0	M34	1.528
233.0	F35	0.509
777.6	M51	1.139
777.6	M52	6.072
777.6	F56	2.131

[1]M = male, F = female

APPENDIX II

PREPARATION OF PRAIRIE DOGS FOR THE SECONDARY TOXICITY STUDY

INTRODUCTION

Black-tailed prairie dogs were fed warfarin bait for the secondary toxicity test because they represent a typical model of a target vertebrate pest in which warfarin baits could be used to manage their population numbers. The treated prairie dogs were fed to domestic ferrets in an effort to determine secondary hazard and then project potential field hazards.

METHODS

Bait Preparation and Analysis

The purpose of this study was to test the secondary susceptibility of domestic ferrets to warfarin-fed prairie dogs

Two formulations of warfarin bait (0 and 500 ppm) were tested. The warfarin technical product (99% purity) was supplied by Sigma Chemical Company, St. Louis, Missouri. A 1.0% warfarin concentrate was made to aid in mixing and to obtain a sufficient homogeneity for the warfarin formulations. After each bait ingredient was added, the mixer was operated for 20 seconds to mix the ingredients together. When a large amount of concentrate was added, many smaller portions of the concentrate would be added and then mixed for about 20 seconds. After all of the ingredients were added, the mixer was run for 15 minutes to achieve a homogenous mixture.

The control bait, which contained 0 ppm warfarin, was mixed in the same manner as the other formulations. Ingredients of the bait are not mentioned for reasons of confidentiality.

All baits were analyzed to determine exact warfarin levels. Approximately 40 g samples of bait were collected from each of the 6 formulations for freezer storage stability, animal room stability, and concentration verification testing. Samples were ground using a Udy Cyclone Sample Mill (Udy Corporation, Fort Collins, CO). An organic solvent, methanol, was used to extract the warfarin from the bait matrix. The solvent and the bait were taken through 2 series of sonication, shaking, centrifuging, and decanting, before the warfarin extraction was complete. The analyte was then placed into a High Perfomance Liquid Chromatography (HPLC) vial and analyzed on a HPLC for warfarin concentration. The exact methods of the extraction procedure and analysis are presented in Appendix I.

Test System

All methods used in the study were approved by the Genesis Laboratories, Inc. Institutional Animal Care and Use Committee (Project #96013). Black-tailed prairie dogs were live-trapped on November 4, 1996 from a colony in Larimer County, Colorado. Tomahawk live traps (Tomahawk Live Trap Company, Tomahawk, Wisconsin) were set near the burrow openings and worked into the ground to cover the metal bottom with soil. Clean rolled barley was placed on the trigger device as well as a path leading out of the trap for a distance of about 0.5 meters. Traps were checked at least twice daily.

Prairie dogs were transported to Genesis Laboratories, Inc. (Wellington,

Colorado) in the back of a covered truck in the same trap in which they were captured. All animals were dusted with Adams™ Flea and Tick Dust II (0.5% pyrethrin, SmithKline Beecham Animal Health, West Chester, Pennsylvania), to control ectoparasites.

Individual prairie dogs were placed into a cloth bag and plastic container and weighed using an balance. The maturity of 6 male and 6 female prairie dogs was determined according to body weight (minimum 675 grams and 775 grams for adult females and adult males, respectively) (Hoogland 1995). Preliminary body weight was recorded on the first day of the 5-day exposure period. Final body weights were recorded at test termination or at time of death.

The individual cages had metal screen bottoms with a surface area of 3,720 cm^2 and a height of 41 cm (National Research Council 1992). Prairie dogs received a basal diet of Rodent Laboratory Chow® 5001 (Purina, St. Louis, Missouri), rolled barley, and water *ad libitum*. Bedding and water bottles were changed weekly, while water and feed levels were checked daily. Cages and racks were not cleaned during the study because handling of the animals could cause lesions, bruises, or injury that could bias mortality estimates (Penumarthy and Oehme 1978).

Acclimation of Prairie Dogs

The prairie dogs were held in an acclimation period for 7 days before dosing. Four days after the dosing began, another 3 females and 2 males were added to the 5-day exposure test to ensure there were enough animals for the ferret-exposure test. The prairie dogs were placed in the same animal room and identical cages. These animals

were also acclimated for 7 days.

Environmental Monitoring

Minimum/maximum temperature and humidity in the animal room in the prairie dog and ferret rooms were recorded daily during the entire holding period with a calibrated digital hygrothermometer. The temperature and humidity of the prairie dog study room was maintained between 19 to 25°C and 11 to 43%, respectively. The temperature and humidity of the domestic ferret study room was maintained between 14 to 27°C and 10 to 80%, respectively. Fluorescent lights were on 12 hours per day (0600-1800 hrs.) and regulated by a Paragon Electric Model P101 timer.

The mean maximum and minimum temperature during the test period differed slightly more than 1°C compared to the mean temperatures for the acclimation period. The mean humidity differed slightly more than 4% on the maximum and minimum for the same periods.

Toxicity Test for Prairie Dogs

The prairie dogs were visually inspected by a veterinarian before the presentation of the bait on November 11, 1996 to ensure suitable health for the test. Prairie dogs were reweighed to ensure accurate aging.

Seventeen prairie dogs (8 males and 9 females) were fed 500 ppm warfarin bait for 5 days in a no-choice situation. The warfarin bait was presented in stainless steel metal feed cups attached to a 30 X 30 cm sheet of particle board to catch spilled feed. A flat circular fouler with 10 mm diameter holes was placed over the bait to limit spillage.

Seventy grams of bait were presented daily to the prairie dogs. Bait consumption was measured daily, and the consumed portion of bait was refilled with the respective fresh bait. After the 5-day exposure period, the prairie dogs were fed the basal laboratory diet of Rodent Laboratory Chow® 5001 (Purina Mills Inc., St. Louis, Missouri), rolled barley, and water *ad libitum*. Fourteen untreated prairie dogs were euthanized by asphyxiation with CO_2 and frozen for future use in the pre-conditioning for all ferrets and exposure period for the control ferrets.

The prairie dogs were euthanized by asphyxiation with CO_2 after the 5-day exposure period and a 2-day post-treatment observation period, and immediately placed in the freezer for 4 days until the ferret secondary toxicity test. The prairie dogs were observed only twice daily during the feeding test and post-test observation periods to limit stress to the animals which could induce mortality (Penumarthy and Oehme 1978).

Body weights were recorded at the first day of the observation period and the last day or at death to assess weight loss or gain. Weights were taken by holding the prairie dog in a tared plastic container and cloth bag and then recording a reading from a calibrated Ohaus balance.

Tissue analysis

Two of the treated prairie dogs, M (female) and Q (male) were randomly chosen from the freezer to be analyzed for whole body residues of warfarin. This analysis would show the amount of warfarin accumulation in these two prairie dogs and show that the prairie dogs in this test were positive for warfarin.

The extraction procedure entailed thawing the frozen prairie dogs in warm water,

blending with an equal amount of acetone, and the taking 3 - 5 g samples from the homogenous mixture. Samples were diluted with acetonitrile and minced in a Tissumizer to homogenize the sample further. The sample was centrifuged, and then the analyte was decanted into a clean centrifuge tube. The extraction procedure was performed twice. The extracts were combined and then filtered into a HPLC vial for analysis. Details on the methods and extraction procedure of the analysis are given in Appendix I.

RESULTS AND DISCUSSION

Prairie Dog Feeding Test

The daily warfarin bait consumption of each prairie dog and the mean is presented in Table 1. The table illustrates the different consumption rates of males and females. One prairie dog (D, male) was found dead within the 5-day feeding period and 3 prairie dogs (E, M male; J, female) were found dead during the 2-day post-test observation period. All other prairie dogs exposed to the bait showed no signs of warfarin poisoning because they did not have time for the bait to activate.

Prairie dogs M (female) and Q (male) had 0.400 and 0.595 ppm of warfarin accumulation, respectively. The warfarin found in their tissues is 1.0 and 0.9% of body weight respectively, of what they originally consumed during the exposure period.

The tissue method also offers a reliable, reproducible, and rapid analysis of test substances in tissues. High CV values are due to the difficult tissue matrix of a whole prairie dog. The tissue results from analysis were much lower than the calculated concentration ingested by the ferrets. This factor helps decrease the possibility of secondary deaths if warfarin is quickly decomposed after consumption.

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Table 1. Consumption of 500 ppm warfarin bait by prairie dogs and the calculated amount of warfarin in the prairie dogs without degradation of the warfarin. These prairie dogs were prepared for use for the secondary hazard test.

Prairie Dog Identification & Sex	Total Consumption (g)	Estimated Warfarin in Prairie Dog (mg)
A ♂	139.2	69.6
B ♂	174.0	87.0
C ♂	190.1	95.1
D ♂	76.2	38.1
E ♂	134.5	67.3
F ♂	34.8	17.4
P[1] ♂	90.7	45.4
Q[1] ♂	157.8	78.9
G ♀	2.6	1.3
H ♀	85.5	47.8
I ♀	85.2	47.6
J ♀	63.4	31.7
K ♀	106.2	53.1
L ♀	104.8	52.4
M[1] ♀	55.9	27.9
N[1] ♀	126.8	63.4
O[1] ♀	30.6	15.3
Mean ♀ Consumption	73.4	37.8
Mean ♂ Consumption	124.7	62.4
Mean Total Consumption	97.5	49.4

[1]The exposure period for these prairie dogs was started 4 days after the others.

APPENDIX III

THE POTENTIAL OF SECONDARY POISONING OF BLACK-FOOTED FERRETS WHEN EXPOSED TO BLACK-TAILED PRAIRIE DOGS FEEDING ON A WARFARIN BAIT

INTRODUCTION

Black-footed ferrets (Mustela nigripes) are one of North America's most endangered species (Cahalane 1954, Snow 1972, Hillman and Clark 1980). Careful practices were taken to assure the population would persist, but the population continued to dwindle in the mid-1980s because of loss of prairie dog (Cynomys spp.) range and from canine distemper (Fagerstone 1987). The last remaining 25 ferrets were captured and brought into captivity in an effort to protect the remaining individuals from predators and breed them in captivity. Research in the holding facility provided life history and behavioral information difficult to gain in the field (Fagerstone 1987).

Black-footed Ferret Recovery Team Proposal

The first line of defense to any poisoning of black-footed ferrets are surveys of prairie dog colonies. The United States Fish and Wildlife Service (USFWS) conducts surveys to determine whether ferrets are present on the inspected area. If any signs of ferret presence are observed, poisoning and other disturbances of the prairie dog colony will be prohibited. Black-footed ferrets typically frequent large prairie dog colonies (> 40 ha). Larger colonies are recognized as having greater potential for ferret habitat according the Black-footed Ferret Recovery Team Proposal (Fagerstone 1987).

United States Executive Order 11643 prohibited the use of strychnine and compound 1080 on Federal Lands since 1972 (Fagerstone 1987). Some poisoning of prairie dogs still occurs on private, state, and federal lands, with the use of other registered toxicants. If prairie dog colonies are allowed to expand on the federal lands, both the prairie dog and the black-footed ferret recovery could be enhanced, and ensure populations will survive for the future.

Feeding habits

Black-footed ferrets are known for their dietary preference for prairie dogs. Sheets et al. (1972) excavated 17 black-footed ferret burrows and recovered 72 scats in which 86% of the animal matter was prairie dog remains, and 14% mouse species (Peromyscus spp.). Captive black-footed ferrets have been reported to consume diets including prepared mink diets, dog food, birds, and various rodents (Aldous 1940, Erickson 1973). Captive juvenile black-footed ferrets ate dead prairie dogs, mice, ground squirrels (Spermophilus spp.), and eastern cottontails (Sylvilagus floridanus) (Hillman 1968). Summaries of early naturalists' report ferrets eating reptiles, birds, insects, mice, ground squirrels, and rats (Henderson et al. 1969). These examples show that while the black-footed ferret almost solely eats prairie dogs, they will eat other mammal, avian, reptilian, and insectivorous foods in the wild to supplement their diet or through opportunistic feeding.

The eating habits have been recorded for many of the mustelids including the black-footed ferret. A captive black-footed ferret was seen feeding upon a black-tailed prairie dog (C. ludovicianus) by eating the entire neck and posterior skull area from the

ventral side (Progulske 1969). These habits are similar to ones seen during the test and from other accounts of weasels (Mustela spp.). For example, the long-tailed weasel (M. frenata) eats prey by consuming the head and maybe the brain first, then continuing with the internal organs and surrounding body, working to the posterior end. The skull, skin and tail are the only uneaten portions (Moore 1945, Fitzgerald 1977, DeVan 1982). Fitzgerald (1977) also noticed that the stomach, skull, feet and tail were not eaten and discarded at the entrance of the nest. The short-tailed weasel (M. ermina) and the least weasel (M. nivalis) have also been reported discarding the stomach, but sometimes eating the intestines (Day 1968). This is an important note. The chances of ferrets becoming poisoned by direct consumption of undigested warfarin bait is reduced if the stomach is discarded. Then the only chance of poisoning would be by secondary exposure. Warfarin is known to be concentrated in the liver, but measurable amounts also occur in the adrenal glands, lungs, bone marrow, kidneys, and lymph nodes (Machlin 1984). During this study, the domestic ferrets were eating some of the more consequential organs of the prairie dogs which have the ability to accumulate the toxin, but they showed no signs of intoxication.

The least weasel always begins by eating the entire brain and head first, then proceeds towards the posterior until all of the mouse is consumed (Allen 1940, Heidt 1972). If more than 1 mouse was present, the least weasel would eat the head and brain first of all available animals and then proceed to finish eating 1 of them (Llewellyn 1942). According to Criddle and Criddle (1925), all weasels kill by penetrating the skull with their teeth or by cervical dislocation. They also stated that weasels practice surplus killing during late summer to provide food for the upcoming winter months.

Mustelids appear to show no preference for particular organs, but rather a pattern of tissue consumption. When more than 1 prey animal is present, it appears that an innate instinct to kill predominates, instead of consuming 1 at a time, as needed. When this happens, carcasses will be stored as surplus food, and over time the probability of secondary poisoning from warfarin would greatly decrease because of the short half-life (42 hours) (Ford 1993). Even if animals are killed as needed, the chances of secondary poisoning will be remote because of the time it takes to capture, consume, and become hungry for another animal. The domestic ferrets of the secondary hazard study took 4 days to consume an entire prairie dog. During this time, warfarin is degrading in the gastro-intestinal system of the prairie dogs, decreasing the chance for secondary poisoning.

Consumption by Mustelid Species

Domestic ferrets were used in the secondary hazard study as a model for species of weasels and black-footed ferrets that may be found in prairie dog colonies (Fagerstone 1987). Domestic ferrets have similar consumption rates to the black-footed ferret.

Progulske (1969) reported that a captive black-footed ferret observed for 7 months consumed 60 to 364 g/day, though only on 3 days did the animals eat less than 120 grams. Hillman, unpublished data, reported male and female black-footed ferret consumption for 5 days at 123.2 and 83.7 g/day, respectively (Fagerstone 1987). In my study, domestic ferret male and female consumption during the 5-day secondary warfarin exposure was 155.4 and 68.1 g/day, respectively. The results from the secondary test are similar to the black-footed ferrets, with the domestic males eating 26.1% more prairie dog than the

male black-footed ferrets, and the domestic females eating 18.6% less than the female black-footed ferrets. The consumption from the pre-conditioning period of the secondary hazard study showed dramatic differences from the exposure period. The mean consumption for the males and females was 186.3 and 57.4 g/day, respectively. Some of these differences can be accounted for because this was the first time the domestic ferrets were exposed to raw meat and the female ferrets were slow to begin feeding on the dead prairie dogs in both the pre-conditioning and the exposure periods. The males, however, showed no restraint in eating raw meat. In comparison, the domestic ferrets consume varying amounts of prairie dog per day, like the consumption observations of the black-footed ferrets during the 7-month Progulske (1969) study, but the total amount consumed was similar with both species.

Aulerich et al. (1987) reported mink (M. vison) as being not susceptible to secondary warfarin poisoning after exposure for 4 weeks on contaminated New Zealand white rabbit carcasses (22.5 ppm). Consumption of warfarin was calculated at 2.99 mg warfarin/day for 28 days, and 100% survival was achieved with no physical observations of warfarin toxicosis. The male and female domestic ferrets in the Genesis Laboratories, Inc. study consumed a mean of 18.64 and 8.14 mg warfarin/day, respectively. Even though the domestic ferrets consumed more warfarin over a shorter period, they still did not show any signs of warfarin toxicosis.

Least weasels are considered at high risk from the secondary consumption of warfarin-poisonied individuals because they have high metabolic rates, high food intake rates, and high susceptibility to cold weather stress (Townsend et al. 1984). The secondary consumption of warfarin-poisoned mice caused death in least weasels at a

feeding rate of approximately 0.3 ppm/day for 10 days (Townsend et al. 1984). Although least weasels commonly kill mouse-sized species, and prairie dogs are most likely too large to kill, least weasels have the potential to scavenge upon a dead prairie dog. If least weasels were exposured to a warfarin-poisoned carcass, and maintained a minimum daily intake of 0.3 mg warfarin/kg body weight for at least 12 days, death could occur (Townsend et al. 1984).

Though these mustelid species hunt in their own niche, their ranges do occasionally overlap. The larger mustelids exhibit similar habits in feeding and the amounts they consume. This study and others have shown that the susceptibility of these larger mustelids is difficult to achieve.

Energy Expenditure

Weasels and ferrets are very effective carnivores because of their ability to pass through almost any burrow system to catch a variety of rodents and other animals, some much larger than themselves (Fagerstone 1987). Their elongated body (surface area to weight ratio) and poor insulation allows for heat loss, therefore, ferrets and weasels have compensated with a high metabolism (Scholander et al. 1950). Metabolic rates of adult weasels are 2 to 3 times as high as other animals of the same weight (Scholander et al. 1950, Brown and Lasiewski 1972, Stromberg et al. 1983). A high metabolism means these mustelids must feed often to maintain themselves. Gad and Chengelis (1988) reported that domestic ferrets eat to the limit of their caloric needs and then halt feeding until food is again needed. In addition, they reported that ferrets pass fiber through their system in 3 to 4 hours.

To estimate metabolic needs, Stromberg et al. (1983) estimated the number of prairie dogs a female black-footed ferret must consume to maintain herself during gestation, lactation, growth of young, and self-maintenance after litter dispersal. They estimated the consumption rates from reports of other mustelids. Their model predicted that a female black-footed ferret must consume on average 214 black-tailed prairie dogs/year. A population of 766 prairie dogs would be required for maximum sustainable yield following a logistic growth pattern (intrinsic rate of increase = 1.5) (Caughley 1977). Henderson (1990) reported that black-footed ferrets kill between 150 and 200 prairie dogs/year. With an average of 15 black-tailed prairie dogs/hectare (King 1955, Koford 1958, Tileston and Lechleitner 1966, Stromberg et al. 1983), 51.1 hectares of black-tailed prairie dogs would be needed to support 1 female black-footed ferret, and her young before litter dispersal, for 1 year.

The most susceptible period for secondary poisoning for a female ferret would be during lactation, when she is providing energy for herself from prairie dogs and milk for the litter. She would be most active in finding food on these days (days 136-175) (Henderson et al. 1969). This model assumes pregnancy on day 91 of the year, and gestation from day 91 through 135. Gestation has lower metabolic requirements in comparison to the lactation period, therefore, lower consumption rates. From weaning to litter dispersal, day 176-239 (Henderson et al. 1969), she maintains herself with food and brings food to the young for their maintenance and production requirements (Stromberg et al. 1983). She has high metabolic needs for maintenance because of the increased activity, but not all of the food is being consumed by herself, and not to the extent compared to the lactation period (Henderson et al. 1969).

Powell et al. (1985) tracked a black-footed ferret during the months from December to March, and recorded 20 kills of black-tailed prairie dogs or an average of 5 prairie dogs/month or 1 about every 6 days. Late winter to early spring would be the primary time for warfarin bait application, depending upon climate. If the black-footed ferrets were eating prairie dogs at this slow rate, it would be a similar consumption rate to the domestic ferrets of the study, and all these ferrets survived. Since their consumption rate is low, the warfarin would have time to metabolize further, reducing the chances for secondary deaths.

A "Worst Case Scenario"

Female black-footed ferrets that are maintaining themselves and their young, may require 6 times the amount of food than other adults (Powell et al. 1985). If their consumption rate is increased, they would have the greatest potential for secondary poisoning, assuming that bait would be applied within their home range. Consumption during the lactation period would be most hazardous, because of their increased maintenance and production requirements for themselves and the young (Stromberg et al. 1983), therefore, requiring higher consumption of prairie dogs or other prey.

To achieve a "worst case scenario" we need to look at the period of bait application. Warfarin baits for the control of prairie dogs would most likely be applied in March and April. After this period, control of the prairie dogs could be expected to be reduced because of available alternate feeds. Then, bait application would not encompass the lactation period, eliminating a critical time of poisoning. Instead, the baiting would encompass the pre-gestation and gestation periods, a time of decreased energy

requirements compared to the lactation period.

Warfarin residues in prairie dogs, if fed 500 ppm bait during these periods (61 days of March and April), would be approximately 1 to 4 ppm (Table 6 from Appendix I, Analytical Methods). In the "worst case scenario," the female ferret would eat a fresh prairie dog approximately every day for 61 days. This would seem to be a substantial amount of warfarin per day (approximately 2 ppm/day), but 2.99 ppm/day of warfarin fed secondarily to mink for 28 days resulted in 0% mortality (Aulerich et al. 1987). Also, least weasels survived for 29 and more than 92 days on an average of 1.58 ±0.10 ppm of warfarin secondarily (Townsend et al. 1984). Mink and black-footed ferrets are of similar weights and size (Fagerstone 1987), therefore, I would doubt that the black-footed ferret would be any more susceptible to the warfarin than the mink.

Conclusion

Direct experiments have never been performed upon the black-footed ferret because of their "endangered species" status. Extrapolation of data from surrogate species will always produce some error because of the different species involved, but this is the only degree of safety which can be logically construed. The mink and black-footed ferret can be assumed as acceptable surrogates for each other because they are of similar weight and size. Another close relative, the domestic ferret, has similar consumption rates and food habits as the black-footed ferret.

Some similarities of the species of mustelids are evident in their feeding habits, energy expenditure, and consumption rates. First, the feeding habits of the domestic ferrets are similar to other mustelids. The mustelids usually consume the head and neck

regions first, and then consume the rest of the body as they work towards the tail. Second, energy expenditure is similar within mustelids because of their elongated body shape and poor heat conservation. Finally, carcass consumption of the domestic ferrets was compared to examples of black-footed ferrets in captivity and in the wild.

Behavior and physical characteristics of the domestic ferret and the black-footed ferret are similar to other large mustelids. Their similarities imply that their reaction to warfarin-poisoned prairie dogs would be similar. We have seen in the results of 2 secondary hazard studies having similar results--no mortality. With the information at hand and comparisons made, I would declare that secondary toxicity of warfarin to black-footed ferrets would be no worse than secondary toxicity to mink or domestic ferrets. In short, I believe secondary toxicity of warfarin to black-footed ferrets would be exceedingly difficult to achieve.

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